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ALÉRGENOS DEL HUEVO: DIGESTIÓN GASTROINTESTINAL, INMUNORREACTIVIDAD Y MECANISMOS DE DESENSIBILIZACIÓN

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INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC-UAM)

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Memoria presentada por:

Gustavo Martos Sevilla

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Trabajo realizado bajo la dirección de:

Dra. Rosina López-Alonso Fandiño

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ROSINA LÓPEZ-ALONSO FANDIÑO, PROFESORA DE INVESTIGACIÓN DEL CSIC, Y ELENA MOLINA HERNÁNDEZ, CIENTÍFICA TITULAR DEL CSIC, DEL INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

INFORMAN:

Que el presente trabajo titulado "**Alérgenos del huevo: digestión gastrointestinal, inmunorreactividad y mecanismos de desensibilización**" y que constituye la Memoria que presenta el licenciado en Bioquímica Gustavo Martos Sevilla para optar al grado de Doctor, se ha realizado bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM).

Y para que conste firmamos el presente informe a 9 de enero de 2012.

Fdo.: Rosina López-Alonso Fandiño

Fdo.: Elena Molina Hernández

A mis padres y a mi hermano

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ÍNDICE

RESUMEN

La alergia al huevo es una de las causas más frecuentes de hipersensibilidad inmediata a los alimentos en los países industrializados, sobre todo durante la infancia. La ubicuidad del huevo en los alimentos supone un gran riesgo de ingestión accidental para las personas alérgicas, que deben llevar una dieta muy restringida. No se conocen las características que convierten a una proteína alimentaria en un alérgeno, aunque la capacidad para resistir la degradación gastrointestinal es una propiedad frecuente que permite que los alérgenos puedan ser reconocidos por el sistema inmunitario y desencadenar los síntomas clínicos en individuos susceptibles.

En la presente tesis, se estudia la digestibilidad de distintos alérgenos del huevo empleando un modelo *in vitro* de digestión gastrointestinal fisiológicamente relevante, puesto que tiene en cuenta las condiciones específicas presentes a lo largo del sistema digestivo, tales como acidez, concentración de enzimas digestivas y surfactantes. De esta forma, se ha podido observar que el pH gástrico tiene un efecto determinante en la estructura de los alérgenos ovalbúmina (OVA) y lisozima (LYS), influyendo en su digestibilidad enzimática. Ambos resisten parcialmente la degradación por pepsina al pH típico del estómago adulto en condiciones de ayuno, pero a pHs ligeramente superiores, como los existentes en niños o personas con la función gástrica alterada, la proteolisis es más restringida. Se ha comprobado que los surfactantes naturales, sales biliares y fosfatidilcolina, interaccionan con OVA y LYS durante el proceso digestivo, favoreciendo la hidrólisis enzimática del primero y dificultando la del segundo, que además pierde solubilidad a causa de las sales biliares. Estos datos aportan una función adicional a la bilis, que además de facilitar la digestión de grasas podría incrementar la susceptibilidad de ciertas proteínas al ataque enzimático, aunque en otros casos puede producir efectos inesperados, como la precipitación proteica. Igualmente interesante ha sido advertir que la concurrencia de otros componentes de la matriz del alimento, estudiando la digestibilidad de la clara y la yema conjuntamente, evita la precipitación de la LYS por las sales biliares y podría conducir a que a una mayor proporción de esta proteína alcanzase la parte inferior del intestino.

La búsqueda de nuevas proteínas minoritarias potencialmente alergénicas representa un desafío complicado puesto que el huevo es un fluido biológico muy complejo, pero a su vez resulta importante, en vista de que incluso minúsculas cantidades de alérgeno pueden provocar síntomas graves en personas sensibilizadas. En este trabajo se han identificado de forma tentativa dos proteínas minoritarias en la clara de huevo, ovoinhibidor y clusterina, capaces de reaccionar con la

inmunoglobulina E (IgE) del suero de individuos alérgicos. Asimismo, hemos estudiado la digestibilidad e inmunorreactividad de otra proteína minoritaria, la proteína transportadora de riboflavina, cuya degradación enzimática conduce a la formación de dos péptidos resistentes, pertenecientes a la región Leu41 - Trp84 de la secuencia de la proteína, que contienen epítopos de unión a IgE.

El tratamiento térmico a que se someten las proteínas del huevo en ciertos alimentos conlleva la pérdida de su alergenicidad en muchos casos. Con objeto de avanzar en la comprensión de este fenómeno hemos estudiado también la digestibilidad e inmunorreactividad *in vivo* de los dos alérgenos principales, OVA y ovomucoide (OM), tratados térmicamente. Éstos perdieron su capacidad de producir síntomas en ratones sensibilizados, siendo el mecanismo responsable la combinación de una mayor susceptibilidad de la OVA calentada a la digestión y una menor absorción y capacidad de activación de basófilos de las proteínas tratadas térmicamente.

Por último, se ha profundizado en la base inmunológica de la inmunoterapia oral, la aproximación empleada con más frecuencia en ensayos clínicos para el tratamiento de pacientes alérgicos al huevo. Se administró oralmente inmunoterapia con clara de huevo u OM calentado a ratones sensibilizados para evaluar su efectividad y los cambios generados en el sistema inmunitario. Confirmamos que la inmunoterapia es efectiva para inducir la desensibilización temporal pero no la tolerancia en el modelo murino ensayado. No hallamos supresión de la activación de basófilos y mastocitos periféricos en ratones que habían recibido la inmunoterapia, aunque eran asintomáticos, lo que sugiere que el mecanismo de desensibilización se localiza en la mucosa gastrointestinal y podría estar relacionado con la reducción observada en la expresión de varios genes del epitelio intestinal. Asimismo, pudimos comprobar que la inmunoterapia con OM calentado es igualmente eficaz, postulándose el uso de alérgenos calentados no reactivos como una aproximación válida y más segura para la inmunoterapia oral en humanos, a veces desaconsejada por la alta incidencia de reacciones adversas.

SUMMARY

Egg allergy is a major cause of food hypersensitivity in western countries, especially among children. Allergic patients are put on a restricted egg-free diet but they are still at high risk of accidental exposures. Features that make a food protein an allergen are unknown, although resistance to gastrointestinal digestion is a commonly shared property that enables allergens to be recognized by the immune system and trigger an allergic response in susceptible individuals.

In the present thesis, egg allergens digestibility has been studied by using a physiologically relevant digestion model that takes into account the specific conditions found along the gastrointestinal track, such as acidity, concentration of digestive enzymes and surfactants. Thus, the pH has been found to influence ovalbumin (OVA) and lysozyme (LYS) structure, affecting their digestibility by enzymes. Both allergens resisted partially digestion at a gastric pH typical of an adult fasted state, but proteolysis was more limited at higher pH values, similar to those found in children or in adults with impaired gastric function. The natural surfactants phosphatidylcholine and bile salts were capable of interacting with OVA and LYS during the digestion process, favouring hydrolysis of the former and protecting the latter, which became insolubilized by bile salts. These data suggest an additional role for the bile in the duodenum, not only facilitating lipolysis but also increasing the susceptibility of certain proteins to enzyme degradation, although it could also induce unexpected precipitation of other proteins. Interestingly, when studying egg white and yolk digestion, LYS precipitation was abrogated in the presence of the yolk matrix, which could result in a higher proportion of protein reaching the lower intestine *in vivo*.

Egg is a complex biological fluid and the search for potential minor allergens is a difficult but important task, since minute amounts of allergen are usually enough to provoke an allergic reaction in sensitized individuals. In this work, two low-abundance proteins were tentatively identified in egg white, ovoinhibitor and clusterin, which were able to react with immunoglobulin E (IgE) present in the serum from egg-allergic patients. Furthermore, we studied the digestibility and immunoreactivity of another minor protein,

riboflavin binding protein, whose enzymatic degradation led to the appearance of two resistant peptides containing IgE-binding epitopes within the region Leu41 – Trp84 of the protein sequence.

Thermal treatment of egg proteins frequently results in the loss of their allergenicity. In order to further understand this phenomenon, we investigated heat-treated OVA and ovomucoid (OM) digestibility and immunoreactivity *in vivo*. Both were unable to evoke symptoms in sensitized mice, being the mechanism responsible a combination of an increased susceptibility of heated OVA to hydrolysis and an impaired absorption and reduced basophil activation capacity of the heat-treated proteins.

Finally, this thesis dealt with the immune basis of oral immunotherapy, a widely used approach for treating egg-allergic patients in clinical trials. Sensitized mice were orally administered egg white or heated OM as immunotherapy to investigate the immunological changes induced and their effectiveness. Temporal desensitization but not tolerance was successfully achieved. Suppression of basophil activation or of peripheral mast cells did not occur in treated mice despite being asymptomatic, which points to a local desensitization mechanism within the gastrointestinal mucosa that could be associated with the observed downregulation of several intestinal epithelial genes. Moreover, we confirmed the efficacy of heated OM to produce desensitization, postulating the use of heated, non-reactive allergens as a valid and safer strategy for oral immunotherapy in humans, sometimes discouraged because of the risk of adverse reactions.

LISTA DE ABREVIATURAS

α-La: α-lactalbúmina.

β-Lg : β-lactoglobulina.

Caco-2: Células de adenocarcinoma de colon humano.

ELISA: Ensayo de inmunoabsorción ligado a enzima.

FcεRI: Receptor de alta afinidad para IgE.

IFN-λ: Interferón gamma.

IgA: Inmunoglobulina A.

IgE: Inmunoglobulina E.

IgM: Inmunoglobulina M.

IL-10: Interleuquina 10.

IL-13: Interleuquina 13.

IL-4: Interleuquina 4.

LYS: Lisozima.

OM: Ovomucoide.

OVA: Ovalbúmina.

OVT: Ovotransferrina.

PC: Fosfatidilcolina.

RBfP: Proteína transportadora de riboflavina.

SGF: Fluido gástrico simulado.

TCR: Receptor de la célula T.

TGF-β: Factor de crecimiento transformante beta.

OBJETIVO Y PLAN DE TRABAJO

La alergia al huevo es la segunda causa más frecuente de reacciones adversas a los alimentos, afectando a un 1.3% de los niños de corta edad. Aún no se conocen las características que convierten a una proteína en alergénica, pero se acepta que la capacidad para sobrevivir a las condiciones del sistema gastrointestinal es un elemento clave tanto en la sensibilización como en el desencadenamiento de los síntomas alérgicos. En un estudio pionero[1] se estimó la resistencia a la digestión con pepsina (fluido gástrico simulado, SGF) de varias proteínas alergénicas, empleando un ensayo originalmente ideado para evaluar la biodisponibilidad de aminoácidos. Se encontró que los principales alérgenos del cacahuete, soja, huevo y leche eran más estables que otras proteínas vegetales no alergénicas. Posteriormente, diversos autores confirmaron la mayor estabilidad relativa de los alérgenos aunque los tiempos de resistencia en SGF no siempre coincidían.[2] Por otra parte, muchas proteínas no alergénicas eran también estables en SGF u originaban fragmentos peptídicos resistentes durante más de 60 minutos.[3] Esta divergencia de resultados puede atribuirse, al menos en parte, a la variabilidad en las condiciones empleadas por los distintos laboratorios, referentes al pH, las relaciones enzima / proteína o los métodos de detección, a menudo más orientadas a evaluar la seguridad alimentaria de una cierta proteína que a reflejar las condiciones encontradas *in vivo*. Además, estudios más recientes han demostrado la importancia de considerar también la complejidad de los medios de digestión estomacal y duodenal, la participación de otras enzimas digestivas o la interacción con otros componentes, como lípidos y sustancias tensioactivas naturales. Bajo este nuevo panorama, los alérgenos del huevo, a pesar de su importancia, permanecen como proteínas no suficientemente estudiadas, desconociéndose los factores que influyen en su digestión gastrointestinal en condiciones fisiológicamente relevantes, así como la repercusión de la digestión en su alergenicidad final.

El huevo constituye un fluido biológico muy complejo cuyo proteoma está siendo objeto constante de estudio en los últimos años. La descripción del último alérgeno (Gal d 6) data de 2010 y fue hallado en la yema.[4] Por tanto, es probable que existan proteínas,

aún desconocidas, que contribuyan al carácter alergénico del huevo. Además, es necesaria la búsqueda de alérgenos potenciales no descritos entre el amplio repertorio de proteínas, muchas de ellas minoritarias, presentes en la clara de huevo o en la yema. Por otro lado, es conocido que el tratamiento térmico a que se someten las proteínas del huevo en ciertos alimentos conlleva la pérdida o disminución de su alergenicidad. De hecho, aproximadamente un 70 % de los niños alérgicos al huevo toleran la ingestión del alimento en forma calentada. La explicación a este hecho habitualmente se fundamenta en la pérdida de epítopos conformacionales como consecuencia del tratamiento térmico de los alérgenos, pero podrían existir mecanismos adicionales todavía desconocidos. Igualmente, se ignora la trascendencia concreta de dicho calentamiento en su digestibilidad, absorción e inducción de la respuesta inmunitaria.

A pesar de no existir un tratamiento para curar la alergia, la inmunoterapia oral es la aproximación más ensayada con objeto de restablecer la tolerancia. Aunque no es una práctica habitual por la alta incidencia de reacciones adversas, diversos estudios clínicos han logrado con éxito desensibilizar pacientes alérgicos al huevo. No obstante, el mantenimiento de la tolerancia suele ser temporal y requiere una ingesta continuada del alimento para evitar la recidiva. Por otro lado, la inclusión del huevo calentado en la dieta de personas alérgicas suele ser tolerada y a menudo es beneficiosa para la resolución de los síntomas. Por tanto, la demostración de la eficacia de los alérgenos tratados térmicamente para desensibilizar a pacientes alérgicos podría derivar en una alternativa más segura a los protocolos actuales de inmunoterapia oral que emplean el alimento nativo. El avance en la comprensión de los mecanismos implicados en la insensibilización temporal a un cierto alérgeno es clave para implementar terapias más eficaces y entender el equilibrio entre tolerancia e hipersensibilidad.

El objetivo del presente trabajo ha consistido en estudiar la repercusión de la digestión gastrointestinal, simulada en condiciones fisiológicamente relevantes, en la inmunorreactividad de los alérgenos del huevo, así como en la búsqueda de alérgenos potenciales no descritos entre las proteínas minoritarias. Además, se ha pretendido investigar las causas de la reducida alergenicidad del huevo calentado y el mecanismo de desensibilización que tiene lugar durante la inmunoterapia oral.

Para llevar a cabo este objetivo se ha desarrollado el siguiente plan de trabajo:

- Selección y puesta a punto de un modelo relevante de digestión gastrointestinal. Análisis de la digestibilidad e inmunorreactividad de los alérgenos OVA y LYS: influencia del pH, de la relación enzima:sustrato y de los surfactantes fosfatidilcolina y sales biliares.
- Análisis de la digestibilidad e inmunorreactividad de la clara de huevo completa e influencia de la yema.
- Búsqueda de alérgenos potenciales no descritos entre las proteínas minoritarias del huevo.
- Evaluación de la alergenicidad de OVA y OM calentados en un modelo animal de anafilaxis. Ensayos *in vitro* de la digestibilidad, absorción y capacidad de activación del sistema inmunitario de dichos alérgenos.
- Estudio del mecanismo de desensibilización inducido mediante inmunoterapia oral. Eficacia de la inmunoterapia con OM calentado.

1. INTRODUCCIÓN

1.1. Alergia a los alimentos

1.1.1. Visión general

Las alergias o hipersensibilidades a los alimentos se definen como respuestas inmunitarias adversas a ciertas proteínas alimentarias o alérgenos. Esta caracterización las diferencia de otras muchas reacciones adversas de etiología no inmunológica (Fig. 1). Por ejemplo, encontramos las debidas a desórdenes metabólicos (p.ej. intolerancia a la lactosa, galactosemia, etc.), las causadas por el consumo de componentes activos farmacológicamente (p.ej. cafeína) o incluso reacciones no reproducibles de origen psicosomático. Existen, además, reacciones adversas consecuencia de la ingestión de ciertos componentes tóxicos presentes de modo natural en el alimento (p.ej. en setas o en peces escómbridos). También son frecuentes las intoxicaciones alimentarias por microorganismos patógenos que proliferan en alimentos conservados o manipulados inadecuadamente (toxiinfecciones).

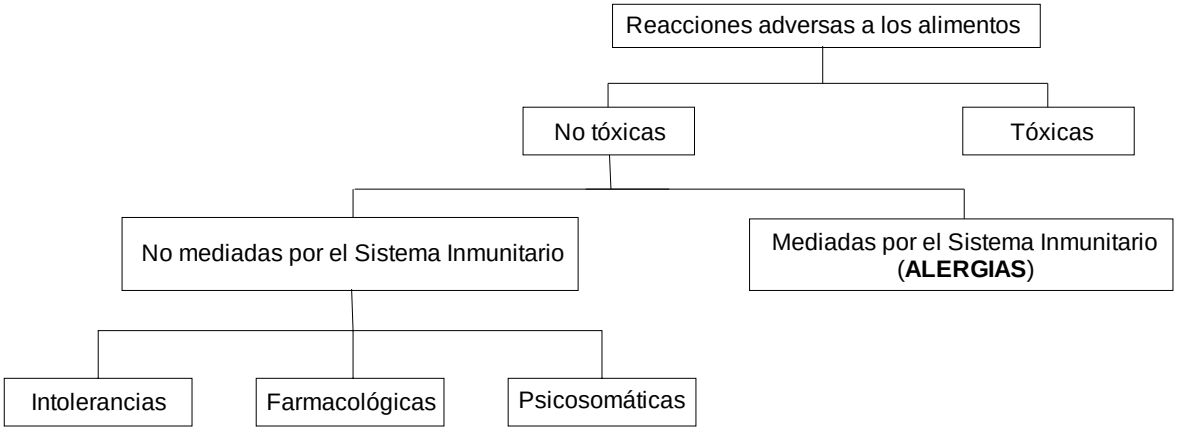


Fig. 1. *Clasificación de las reacciones adversas a los alimentos en función del mecanismo patogénico.*

Cada vez más estudios apuntan hacia un incremento de los casos de alergias alimentarias en las últimas décadas, sobre todo en los países desarrollados.[5, 6] Entre el 3 y el 8% de los niños, y entre el 1 y el 3% de los adultos presenta algún tipo de alergia alimentaria.[7] Los alérgenos varían dependiendo de la localización geográfica y la dieta local. Por ejemplo, la prevalencia de la alergia a frutos secos en EEUU y Canadá está entorno al 1%, mientras que en Singapur y las Filipinas ronda el 0.3%. Y al contrario, los

niños alérgicos al marisco en Canadá suponen alrededor de un 0.5% mientras que alcanzan el 4% en las Filipinas y Singapur.[8] En niños de corta edad, las alergias más comunes son a la leche de vaca (2.5%), huevo (1.3%), cacahuete (0.8%), trigo (0.4%), soja (0.4%), frutos secos (0.2%), pescado (0.1%) y marisco (0.1%). Alrededor del 80% de las alergias a la leche, huevo, soja y trigo se resuelven con la edad, de modo que la población adulta presenta mayoritariamente hipersensibilidad al marisco (2%), cacahuetes (0.6%), frutos secos (0.5%) y pescado (0.4%).[9]

En condiciones fisiológicas normales, los antígenos de la dieta son reconocidos por el sistema inmunitario de la mucosa gastrointestinal que genera un estado activo de inhibición o de tolerancia hacia ese antígeno. Cuando no se induce dicho estado inmunológico o éste se pierde, se produce la sensibilización al alimento en cuestión. La predisposición genética junto a elementos externos tales como la edad de introducción del alimento, la ruta de exposición (digestiva, cutánea o respiratoria), la lactancia materna, la composición de la dieta, la microflora e infecciones intestinales, la presencia de sustancias que alteran el sistema digestivo o potenciadoras de la respuesta inmunitaria, serían los principales factores implicados en la aparición del desorden alérgico.[10] Diversos estudios epidemiológicos han señalado, por ejemplo, que el consumo reducido de ácidos poliinsaturados ω-3, la deficiencia en vitamina D, la introducción tardía de alimentos potencialmente alergénicos o la obesidad, estarían asociados con un mayor riesgo de sensibilización.[11] No obstante, aún quedan por esclarecer los mecanismos de asociación de tales observaciones con el fenómeno alérgico.

También sustentada en estudios epidemiológicos, y con el propósito de explicar el incremento de las alergias en las sociedades occidentales, se ha postulado la hipótesis de la higiene. En esencia, dicha idea sugiere que los hábitos introducidos en las sociedades modernas han privado a los niños de los estímulos inmunológicos necesarios para una adecuada maduración del sistema inmunitario.[12] Cambios en la higiene, la dieta y las prácticas médicas habrían alterado el patrón de exposición a los microorganismos y, en particular, la composición de la microbiota intestinal. De hecho, las interacciones microbio-

hospedador son reconocidas actualmente como un suceso clave en la modulación de la respuesta inmunitaria.[13]

Los trastornos clínicos asociados a las alergias alimentarias pueden afectar a una amplia variedad de sistemas orgánicos.[14] Se pueden manifestar en la piel, como eczema o urticaria; en la piel y mucosa como angioedema; en el tracto respiratorio como edema laríngeo u obstrucción bronquial y posible sibilancia; sistémicamente como anafilaxia, y en el tracto digestivo, desde la boca (síndrome de alergia oral) al ano (proctitis o eczema perianal). La mayoría de los síntomas son inespecíficos, como náuseas, vómitos, diarrea o estreñimiento.

La percepción de padecer algún tipo de alergia alimentaria es generalmente muy superior a la incidencia real de este desorden. El diagnóstico es, por tanto, un primer paso clave para descartar otro tipo de reacciones adversas o patologías. En la práctica clínica, éste se basa en el examen exhaustivo del historial clínico, tests cutáneos selectivos o determinaciones *in vitro* de inmunoglobulina E específica del alimento (si se sospecha que media dicho anticuerpo), dieta de exclusión apropiada y estudios de provocación con el alimento camuflado (provocación doble ciega).[14] Una vez diagnosticada, la única terapia probada es la privación estricta del alimento. Los pacientes han de ser educados para evitar posibles ingestiones accidentales de alérgenos y para saber actuar en caso de una reacción anafiláctica. Las personas con alergias múltiples, especialmente niños, se encuentran en riesgo de sufrir deficiencias nutricionales importantes como consecuencia de sus dietas restringidas. Solo en el caso de lactantes es posible recurrir a fórmulas hipoalergénicas de proteínas de leche de vaca extensamente hidrolizadas o de mezclas de aminoácidos individuales. Por otro lado, son múltiples las aproximaciones terapéuticas que se están explorando encaminadas a restablecer la tolerancia oral al alimento que serán comentadas más adelante en el texto.

1.1.2. Mecanismo inmunológico de la alergia

El sistema inmunitario de la mucosa intestinal ha evolucionado para identificar posibles amenazas en un entorno complejo y variado como es el intestino. La barrera

epitelial intestinal se encuentra continuamente expuesta a multitud de proteínas alimentarias, bacterias comensales y microorganismos patógenos contra los que debe luchar. Existen principalmente dos mecanismos homeostáticos que preservan la integridad de esta barrera: la exclusión inmunitaria por inmunoglobulina A (IgA) y la supresión de respuestas proinflamatorias (Fig. 2).[10] El primer mecanismo controla la colonización epitelial de microorganismos e inhibe la penetración de agentes potencialmente peligrosos. Esta acción la llevan a cabo las células plasmáticas residentes en la lámina propia del subepitelio mediante la secreción de IgA (e IgM) al mucus intestinal. Dichos anticuerpos dificultan la adhesión de microorganismos y activan el aclaramiento de antígenos. Su producción se induce cuando antígenos particulados o microorganismos patógenos son captados por las células M (micropliegues) presentes en las placas de Peyer y entran en contacto con las células inmunitarias. El segundo mecanismo evita la hipersensibilidad, tanto local como periférica, hacia antígenos inofensivos. Así, antígenos solubles inocuos, como por ejemplo proteínas alimentarias, y microorganismos comensales, atraviesan la barrera intestinal y son reconocidos por el sistema inmunitario, desarrollándose la llamada tolerancia oral.

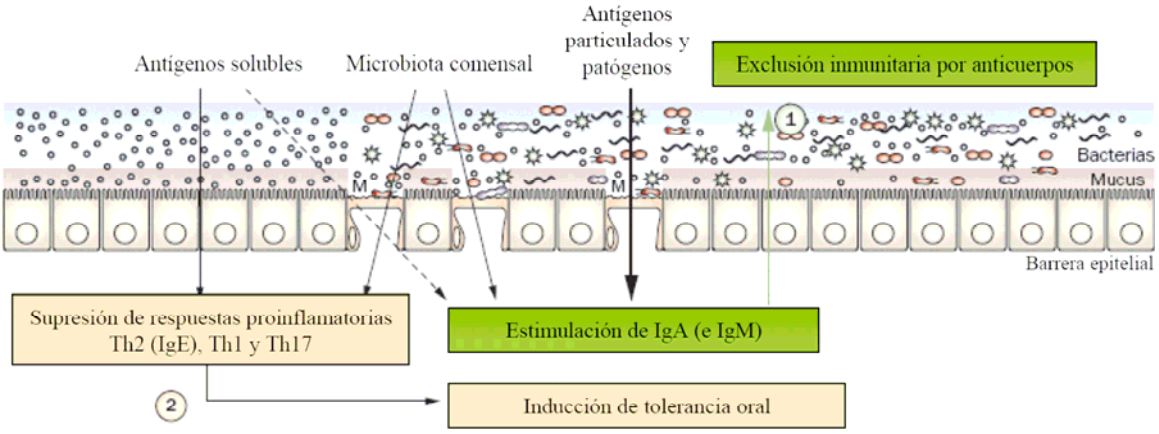


Fig. 2. *Representación de los dos mecanismos homeostáticos principales que preservan la integridad de la barrera epitelial intestinal. Tomado de Brandtzaeg.*[10]

La inducción apropiada de estos mecanismos de homeostasis depende de estímulos exógenos, siendo la etapa neonatal (4 primeras semanas de vida) especialmente crítica para llegar a tal fin. Tanto la barrera de exclusión mediada por IgA como la red de

inmunorregulación requieren un proceso adaptativo. Dicho proceso es muy eficaz en vista de que, aproximadamente, 100 kg de proteínas[15] pasan a través del intestino de un adulto cada año sin causar reacciones adversas. Sin embargo, un fallo en estos mecanismos puede conducir a la pérdida de tolerancia oral y a la aparición de la alergia.

Se especula que cualquier circunstancia capaz de alterar la barrera epitelial, no solo a nivel intestinal, sino también cutáneo o respiratorio, podría ser determinante en la sensibilización a un cierto alimento, sobre todo cuando no se ha establecido previamente tolerancia oral. Por ejemplo, la alergia al cacahuete se ha asociado al uso de cremas infantiles que contienen aceite de cacahuete en niños afectados de dermatitis atópica, enfermedad que ocasiona descamación de la piel.[16] La evasión de la tolerancia oral puede ocurrir también vía respiratoria, como en el síndrome de alergia oral. En este caso, una sensibilización vía respiratoria frente al polen de abedul, que contiene una proteína homóloga a la proteína *Mal d 1* de las manzanas, ocasiona picazón (prurito oral) en pacientes que ingieren manzanas crudas.[17]

No obstante, la ruta gastrointestinal es la vía de sensibilización más común en los casos de alergia alimentaria. Después de sobrevivir en mayor o menor medida a la acidez del estómago, las enzimas digestivas y a la microflora intestinal, los antígenos alimentarios entran en contacto con la barrera epitelial. En este momento, un fallo en los mecanismos homeostáticos antes descritos que regulan la integridad de esta barrera, desencadena una serie de eventos que conducen a la sensibilización. Así como la etapa neonatal es crítica para la correcta inducción de estos mecanismos, la infancia (entre 1 y 5 años de edad) es también crítica para la potencial evasión de éstos. La mayor incidencia de las alergias alimentarias en los primeros años de vida se ha achacado a factores como la menor acidez estomacal, menor concentración de enzimas digestivas, microflora intestinal incompleta, menor cantidad de mucina (constituyente principal del mucus) o una permeabilidad epitelial aumentada. De hecho, estudios donde se neutraliza el pH estomacal demuestran un mayor riesgo de sensibilización,[18] e igualmente ocurre cuando, induciendo situaciones de estrés, se perturban las uniones íntimas entre las células epiteliales, aumentando la permeabilidad intestinal.[19] Dichas circunstancias podrían contribuir a que mayor cantidad

de antígeno inmunológicamente activo confronte la pared intestinal y desencadene una respuesta inadecuada de las células inmunitarias, llevando finalmente a la sensibilización.

En el fenómeno alérgico se pueden considerar dos fases diferenciadas: la fase de sensibilización, en la que el individuo susceptible desarrolla la predisposición inmunitaria a reaccionar contra la proteína alimentaria; y la fase de provocación, donde una sucesiva exposición al alérgeno desencadena los síntomas clínicos. En ambos casos, el acceso del antígeno a la lámina propia del subepitelio intestinal es un episodio común y primario.

1.1.2.1. Entrada del antígeno

La principal ruta de entrada de los antígenos solubles es la ruta transcelular, si bien no se descarta que en el desorden alérgico también pueda ocurrir una relajación de las uniones íntimas entre enterocitos favoreciendo el transporte paracelular, que generalmente está restringido a pequeños solutos (<600 Da) e iones.[20] La ruta transcelular implica primordialmente a los enterocitos, aunque también existen mecanismos de captura directa del antígeno por células dendríticas subepiteliales[21] y de transporte a través de las células M que recubren las placas de Peyer, sobre todo en el caso de antígenos particulados. Estos dos mecanismos adicionales son fundamentalmente no degradativos, mientras que el transporte enterocítico implica la degradación de más del 90% del antígeno transportado.[22]

A pesar de que la absorción intestinal del alérgeno intacto es posible, la mayor parte de éste sufre la degradación por las enzimas digestivas y las enzimas lisosómicas durante el transporte a través del enterocito. Los péptidos que finalmente alcanzan la lámina propia han sido exocitados por los enterocitos en su forma libre o unidos a vesículas que presentan el complejo principal de histocompatibilidad tipo II (MHC-II).[23] También se ha descrito un mecanismo adicional por el que los enterocitos podrían transportar el antígeno intacto unido a la inmunoglobulina E,[24] que cobra importancia en la fase de provocación.

1.1.2.2. Sensibilización intestinal

El lugar donde se inducen todas las respuestas inmunitarias del intestino es el tejido linfoide intestinal, que está constituido por las placas de Peyer, el apéndice y algunos otros folículos linfoides aislados. Las células dendríticas, u otras células presentadoras de antígeno, capturan el antígeno que ha alcanzado la lámina propia, por cualquiera de los mecanismos antes descritos, y migran a los folículos linfoides (Fig. 3). Allí, el antígeno procesado es expuesto a las células T vírgenes (linfocitos T) unido al complejo MHC-II, originando su diferenciación a células Th1, Th2, Th17 o T reguladoras (Treg).[25] Esta activación de las células T puede también producirse en los nódulos linfáticos mesentéricos cuando las células dendríticas que han capturado el antígeno migran a ellos para realizar la presentación del antígeno. La diferenciación hacia uno u otro tipo de célula depende de distintas señales estimulantes (todavía desconocidas) en el dominio de la célula dendrítica, como citoquinas (proteínas reguladoras celulares) y moléculas derivadas de microorganismos. Por otro lado, las células B también tienen la capacidad de reconocer el antígeno, procesarlo y exponerlo en su superficie unido al complejo MHC-II. El encuentro del antígeno provoca la proliferación de clones de células B específicas del antígeno reconocido.

El suceso clave en la alergia alimentaria consiste en la proliferación preeminente de células Th2 tras una primera ingestión del alérgeno.[25] En los folículos linfoides, dichas células interaccionan con las células B que exponen el antígeno unido al complejo MHC-II, induciéndose la producción de citoquinas Th2, mayoritariamente IL-4 e IL-13, que ocasionan la diferenciación de las células B a células plasmáticas productoras de inmunoglobulinas E (IgE) específicas. La diferenciación completa ocurre tras la migración de las células B a los nódulos linfáticos mesentéricos, de donde pasan a la circulación sanguínea para su extravasación final a la mucosa intestinal principalmente (Fig. 3). Los anticuerpos IgE producidos por las células plasmáticas se unirán al receptor FcεRI de mastocitos residentes en la lámina propia y a los receptores de basófilos que circulan en la sangre.

1.1.2.3. Fase de provocación

En una posterior ingestión del alérgeno alimentario, éste accederá a la lámina propia intestinal conservando más o menos integridad estructural, dependiendo de su susceptibilidad enzimática y la ruta de transporte seguida. Allí, el antígeno se unirá a través de algunas regiones de su estructura (epítopos) a como mínimo dos anticuerpos IgE anclados sobre la superficie de mastocitos (Fig. 3),[26] induciendo la desgranulación y liberación masiva de mediadores (tales como histamina, citoquinas y proteasas) que conducen a una variedad de síntomas cutáneos, gastrointestinales, respiratorios o sistémicos (anafilaxia). Al contacto con el antígeno, también se activan las células efectoras Th2, liberando citoquinas responsables del reclutamiento y activación de otras células como eosinófilos y basófilos.

1.1.2.4. Tolerancia oral

En condiciones fisiológicas, existen principalmente tres mecanismos tolerogénicos, basados en la antes mencionada supresión de respuestas proinflamatorias: anergia, delección e inhibición de células T efectoras.[27] La anergia consiste en la inactivación de la célula T cuando encuentra el antígeno pero faltan señales coestimulatorias, como ocurre en ausencia de gérmenes. La delección es un mecanismo de apoptosis que se induce cuando la presentación del antígeno tiene lugar en presencia de ligandos inhibidores. Estos dos mecanismos operan a altas dosis de antígeno.

Cuando la dosis del antígeno es baja, se induce la proliferación de células Treg antígeno-específicas, bien localmente en la mucosa o periféricamente en los nódulos linfáticos o en el hígado.[28] Este subtipo celular inhibe a las células T efectoras mediante citoquinas supresoras como IL-10 y TGF-β. Así, las células Treg inhiben la activación y liberación de citoquinas de todos los subtipos de células T efectoras y son importantes para mantener el equilibrio Th1/Th2,[10] que se encuentra singularmente polarizado en la dirección Th2 en los individuos alérgicos.

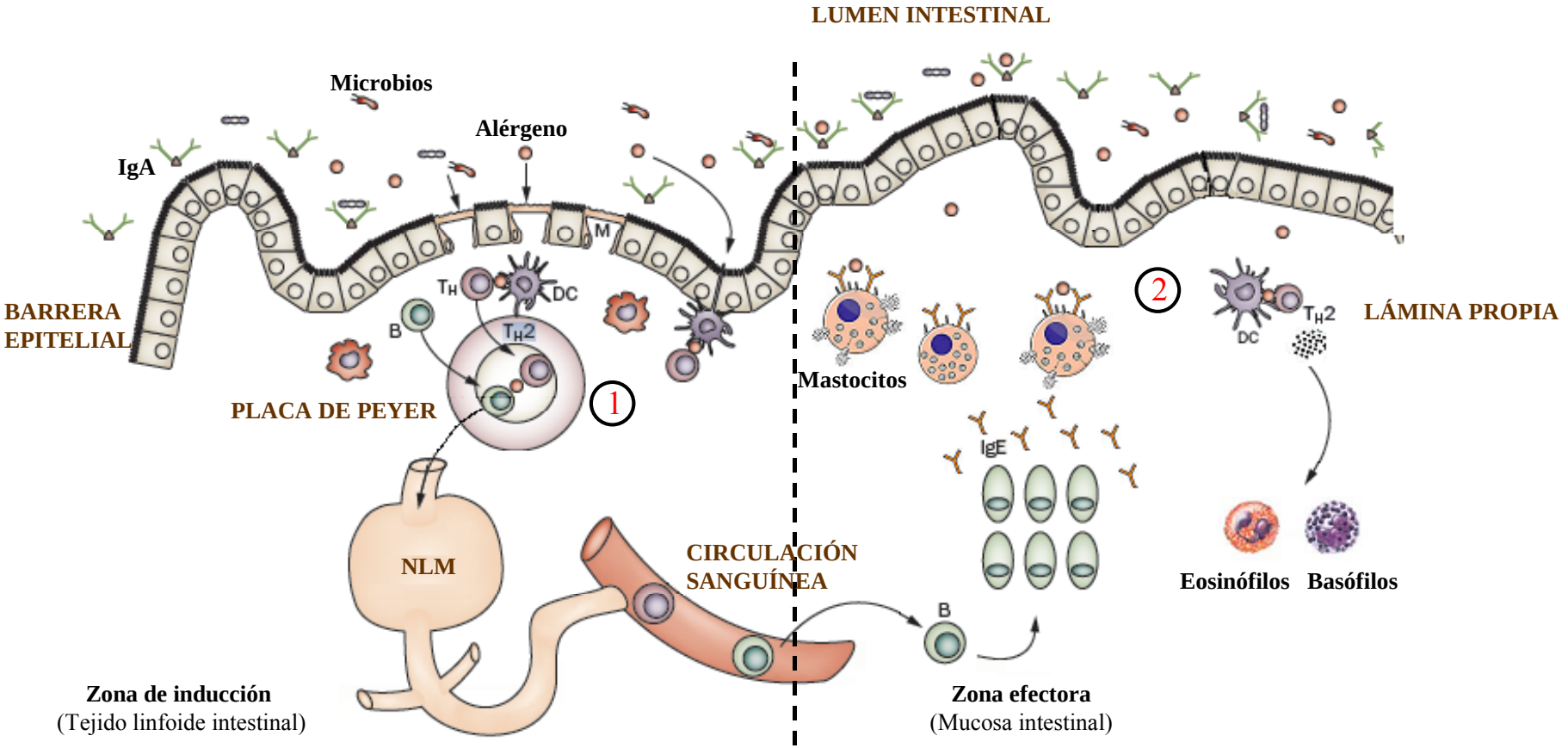


Fig. 3. *Representación de los acontecimientos celulares y moleculares que conducen a la sensibilización y respuesta alérgica. 1) Fase de sensibilización: Las células dendríticas (DC) que han capturado el antígeno lo presentan a las células Th vírgenes provocando su diferenciación a Th2. Éstas interaccionan con las células B en las placas de Peyer u otros folículos linfáticos induciendo su diferenciación a células plasmáticas que migran a través de los nódulos linfáticos mesentéricos (NLM) a la sangre y de ahí a la mucosa intestinal para producir IgE específica que se une a los receptores de mastocitos. 2) Fase de provocación: El alérgeno que vuelve a atravesar la barrera epitelial interacciona con mastocitos produciendo su desgranulación y con células Th2 efectoras que liberan citoquinas que atraen a otras células efectoras como eosinófilos y basófilos. Adaptado de Brandtzaeg.*[10]

Un fallo en estos mecanismos de tolerancia, junto con la presencia de factores que promueven la diferenciación Th2, desencadenan los acontecimientos que llevan a la sensibilización y posterior respuesta alérgica.[29]

1.1.3. Aproximaciones terapéuticas

La gestión clínica actual de la alergia alimentaria se limita a la prescripción de una dieta estricta libre del alimento pernicioso, consejo nutricional y tratamientos de emergencia en caso de reacción adversa. Aunque los primeros intentos para desensibilizar pacientes con alergia a los alimentos datan de hace más de 100 años, no existe hasta el momento ninguna terapia aceptada que haya demostrado acelerar el desarrollo de tolerancia oral o proteger eficazmente contra exposiciones accidentales. Ello es en gran parte debido a que, dada la potencial severidad de los síntomas adversos en los pacientes alérgicos a los alimentos, la relación beneficio/riesgo desaconseja la aplicación de la inmunoterapia en muchos casos. Además, pese a que distintos protocolos de inmunoterapia han logrado cierto éxito, los resultados de desensibilización suelen ser temporales y requieren una ingesta continuada del alimento para evitar la reaparición de la hipersensibilidad.

Los pacientes con alergia alimentaria se pueden dividir en tres fenotipos básicos: alergia transitoria, alergia persistente y síndrome de alergia oral (en personas alérgicas al polen).[30] Parece que cada una de estas formas de alergia mediada por IgE es el resultado de un mecanismo inmunológico diferente y por tanto podría requerir distintas aproximaciones inmunoterapéuticas.

Los casos de alergia transitoria, aquellos que se resuelven hacia los 6-8 años de edad, son los que mejor responden a la terapia, aunque a menudo se arguye que estos pacientes no necesitarían tratamiento. No obstante, los beneficios potenciales de una terapia implican acelerar el desarrollo de tolerancia, mejorando su calidad de vida y nutrición. La alergia persistente, sin embargo, representa un desafío mayor. Estos pacientes suelen responder peor a la terapia, bien no desensibilizándose, necesitando un tratamiento más prolongado o presentando reacciones adversas más graves durante el proceso.

En la actualidad, se están explorando numerosas estrategias terapéuticas dirigidas a los alimentos que más frecuentemente producen reacciones anafilácticas mediadas por IgE (cacahuetes, frutos secos y marisco) o que afectan en gran medida a los niños (leche y huevo). Las aproximaciones son tanto alérgeno-específicas, como no específicas. Entre las primeras se encuentran la inmunoterapia oral,[31, 32] sublingual[33] y epicutánea,[34] en las que se administran, por distintas vías, cantidades crecientes de proteínas nativas para potenciar la respuesta inmunitaria. También en modelos murinos se han ensayado inmunoterapias con proteínas recombinantes[35] (modificadas para presentar reducida capacidad de unir IgE) o conjuntamente con Escherichia coli inactivada por calor.[36] Asimismo, se han empleado dietas con alimentos calentados, como leche[37] o huevo,[38] que permiten una aproximación más segura al desarrollo de tolerancia, ya que el calentamiento generalmente disminuye la alergenicidad de estos alimentos. Respecto a las estrategias inespecíficas, se ha investigado el uso de anticuerpos monoclonales frente a IgE humana,[39] que podría aumentar la dosis requerida para sufrir una reacción alérgica, o la medicina tradicional china,[40] cuya eficacia ha sido demostrada en ratones y se está actualmente ensayando en humanos. También se han ensayado citoquinas,[41] bacterias o agentes víricos inmunomoduladores,[42] o suplementos de prebióticos y probióticos.[43, 44]

1.2. Características de los alérgenos alimentarios

A pesar de la enorme diversidad de la dieta humana, son pocos los alimentos responsables de la mayoría de las alergias alimentarias. En los niños, por ejemplo, el 80% de los casos se deben a la leche, al huevo y a los cacahuetes. Los datos actuales evidencian que las proteínas con actividad alergénica no se encuentran distribuidas al azar entre las miles de familias proteicas conocidas, sino que se restringen a solo unas pocas. Radauer y col.[45] identificaron un total de 29 familias proteicas diferentes que incluían más de un alérgeno alimentario. A pesar de este reducido número de familias, las estructuras y funciones que presentan los distintos alérgenos son dispares y no permiten establecer un nexo común. Entre estas funciones se encuentran la hidrólisis de polisacáridos, la unión y transporte de ligandos, el almacenamiento de reserva o la estructuración del citoesqueleto, entre otras (Tabla 1).

Tabla 1. *Función de las principales familias proteicas a que pertenecen los alérgenos alimentarios. Adaptada de Hoffmann-Sommergruber y Mills.*[46]

Familia proteica	***Función***	***Ejemplos de Alimentos***
Alérgenos vegetales		
Prolaminas	Proteínas de almacenamiento de la semilla, función defensiva, transferencia de lípidos, proteasas o inhibidores de proteasas	Trigo (ω-5 gliadina), cebada, maiz, nuez de brasil (Ber e 1) cacahuetes (Ara h 2), melocotón (Pru p 3), manzana (Mal d 3)
Cupinas	Proteínas de almacenamiento de la semilla	Cacahuetes (Ara h 1), frutos secos, sésamo (Ses I 3), soja (glicinina)
Profilinas	Unión a actina, proteínas reguladoras	Apio, zanahoria, melón, naranja, plátano
Otras: proteínas relacionadas con Bet v 1, Oleosinas, endoquitinasas, β 1,3-glucanasas, proteínas tipo traumatina	Función defensiva, transportador de esteroides, estabilizador de grasas, hidrólisis de quitina	Legumbres, frutos secos, semillas, plátano, aguacate, castaña, kiwi, cítricos, uvas
Alérgenos animales		
Tropomiosinas	Regulan la contracción del músculo	Camarón (pen a 1) y otros crustáceos, moluscos
Parvalbúminas	Unión de Ca^{2+}	Bacalao (Gad c 1) y otros peces, anfibios
Caseínas	Unión de Ca^{2+}	Leche de vaca, cabra y oveja
Lipocalinas	Transporte de moléculas pequeñas	Leche (β-lactoglobulina)
α-lactoalbúmina / Lisozima tipo C	Síntesis de lactosa / función defensiva	Leche de vaca / huevo de gallina
Inhibidores Kazal de serin-proteasas	Inhibidores de proteasas	Huevo de gallina (ovomucoide o Gal d 1)
Serpinas	Inhibidores de proteasas	Huevo de gallina (ovoalbúmina o Gal d 2)
Transferrinas	Transporte de hierro	Leche de vaca (lactoferrina), huevo de gallina (ovotransferrina o Gal d 3)

Asimismo, el hecho de poseer similitud de secuencia con alérgenos conocidos ha permitido explicar la reactividad cruzada de algunas proteínas presentes en alimentos distintos (por ejemplo manzana, pera o cereza en el síndrome de alergia oral). Sin embargo, hasta la fecha no se ha encontrado ninguna propiedad de las proteínas alergénicas que se pueda considerar como causa principal de la alergia. Por otra parte, no solo las propiedades estructurales o funcionales intrínsecas de estas proteínas, sino también factores adicionales

como la abundancia en el alimento, o su estabilidad frente al procesado y la digestión gastrointestinal, pueden contribuir a su alergenicidad final.

1.2.1. Epítopos

Todos los alérgenos alimentarios presentan en su estructura regiones que son reconocidas por el sistema inmunitario y determinan su potencial alergénico. Dichas regiones se denominan epítopos, y pueden ser lineales o conformacionales (Fig. 4). Los primeros dependen exclusivamente de la secuencia primaria de la proteína, por lo que su ruptura solo puede producirse por hidrólisis enzimática. Los segundos comprenden aminoácidos que se disponen espacialmente conformando un lugar de afinidad para la interacción con el sistema inmunitario. Por tanto, como dependen de la estructura secundaria y terciaria de la proteína, son más susceptibles de perderse por desnaturalización o hidrólisis enzimática.

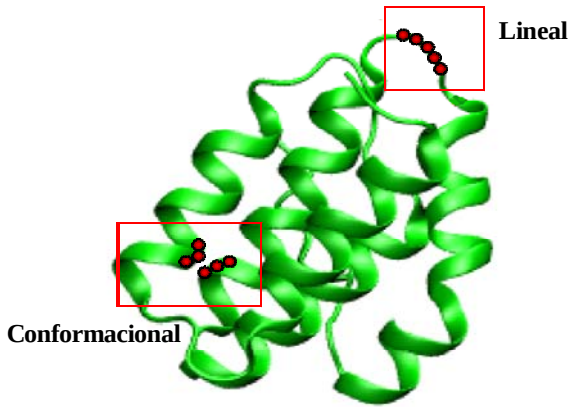


Figura 4. *Representación esquemática de los epítopos lineales y conformacionales.*

Existen epítopos reconocidos por los receptores de las células B y otros reconocidos por los receptores de las células T (TCR). Los primeros determinan la producción de inmunoglobulina E específica de esa región del alérgeno mientras que los segundos intervienen en la activación de las células T, tanto en la etapa de sensibilización como en la de provocación (Fig. 3). Los epítopos distinguidos por la célula B, también denominados epítopos de unión a IgE, constituyen por tanto la región de la proteína reconocida por la IgE anclada al receptor FcεRI de mastocitos y basófilos y que provoca su desgranulación. Huby y

col.[47] afirmaron que un alérgeno debe contener al menos dos epítopos de unión a IgE, de como mínimo 15 residuos de longitud cada uno, para poder activar a los mastocitos y basófilos.

1.2.2. Abundancia en el alimento

La mayoría de los alérgenos son constituyentes proteicos mayoritarios en sus respectivos alimentos, como ocurre en la leche, huevos, frutos secos, soja o trigo. Una posible explicación a este hecho podría ser la mayor probabilidad de que grandes fracciones intactas de la proteína sobrevivan a la degradación gastrointestinal y atraviesen la barrera intestinal, sobrestimulando al sistema inmunitario. Sin embargo, hay algunas excepciones a esta regla, como es el caso del principal alérgeno del bacalao (*Gad c 1*), con una abundancia muy pequeña en el músculo del pez.

1.2.3. Estabilidad y resistencia a la digestión gastrointestinal

La resistencia a la digestión es una característica muy extendida en los alérgenos alimentarios, ya que cuanto más tiempo permanezca intacto el alérgeno, mayor proporción de éste podrá ser absorbido por el tracto gastrointestinal y provocar la respuesta alérgica. En realidad, el alérgeno será capaz de provocar una reacción alérgica mientras conserve integridad estructural suficiente como para mantener sus epítopos reconocibles por la IgE humana. Se ha demostrado que situaciones en las que el proceso digestivo se ve comprometido, como ocurre a pH estomacal elevado en pacientes medicados con antiácidos, existe un mayor riesgo de sensibilización a determinados alimentos.[48] Además, otros estudios han mostrado que la encapsulación de proteínas de la dieta, evitando su degradación durante el tránsito gastrointestinal, puede inducir alergia en ratones[49] e incluso eliminar la tolerancia previamente adquirida.[50]

Un método clásico de evaluar la resistencia a la digestión ha sido el modelo *in vitro* de tratamiento con pepsina a pH ácido. Los principales alérgenos del cacahuete, soja, mostaza, huevo y leche fueron evaluados respecto a su estabilidad frente a la digestión y comparados con proteínas de alimentos no alergénicos.[1] Todos los alérgenos resultaron ser

considerablemente más resistentes a la pepsina. Otros autores demostraron que algunas modificaciones químicas que alteraban la estabilidad de alérgenos de trigo o leche conducían a una mayor susceptibilidad a la digestión por pepsina y a la pérdida de su alergenicidad. [51, 52]

Merece la pena destacar que aunque la resistencia a la digestión es una característica muy extendida entre los alérgenos alimentarios, existen multitud de excepciones en las que proteínas lábiles a las enzimas digestivas mantienen su alergenicidad. Éste es el caso, por ejemplo, de los llamados alérgenos incompletos, incapaces de sensibilizar a un individuo pero sí de ocasionar una respuesta alérgica debido a la homología de secuencia que presentan con otros alérgenos que sí sensibilizan. Así ocurre en el ya mencionado síndrome de alergia oral, en el que las proteínas de la manzana (*Mal d 1*), pera (*Pyr c 1*), albaricoque (*Pru ar 1*) y cereza (*Pru av 1*) son homólogas al alérgeno completo *Bet v 1* del polen. Dichas proteínas, lábiles a la pepsina, son capaces de producir una reacción adversa solo si ha existido una sensibilización cruzada previa con el alérgeno del polen.

Diversos factores, tanto intrínsecos como extrínsecos, tienen una influencia reconocida en la estabilidad y susceptibilidad enzimática final de los alérgenos alimentarios (Fig. 5). Características estructurales como puentes disulfuro, una estructura cuaternaria compacta o la unión de azúcares u otros ligandos han sido asociadas a una mayor estabilidad proteica. Además, componentes de la matriz del alimento en combinación con el procesado al que se somete antes de consumirlo, pueden tener un impacto decisivo en la digestibilidad e inmunorreactividad final de los alérgenos.

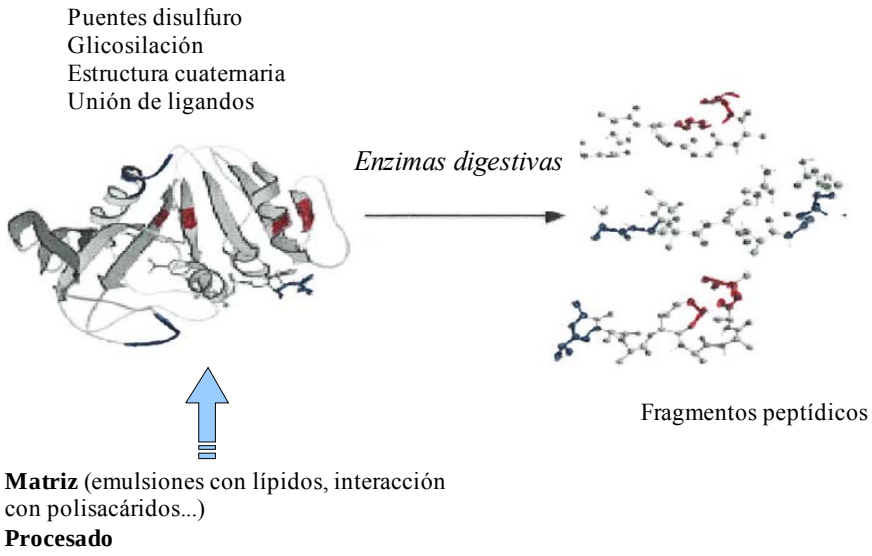


Figura 5. *Representación de algunas de las características y factores implicados alergenicidad final de las proteínas alimentarias.*

Una de las características estructurales claramente relacionada con la estabilidad son los puentes disulfuro. En general, tanto los puentes disulfuro intra- como intercatenarios restringen la estructura tridimensional de modo que la distorsión por agentes químicos o por calor es limitada y a menudo reversible.[53] Además, las aspartil-proteasas como la pepsina requieren un cierto grado de flexibilidad en sus sustratos, ya que acomodan al menos 7 residuos aminoacídicos contiguos que deben hallarse en una conformación desplegada a lo largo del sitio activo del enzima.[54] Por tanto, la compactación y rigidez que aportan los puentes disulfuro dificulta frecuentemente la degradación enzimática. Por ejemplo, las 2S albúminas de la mostaza o de la nuez de brasil, pertenecientes a la familia de las prolaminas, presentan estructuras compactas debido a 4 puentes disulfuro y exhiben alta resistencia al calor, pHs extremos y proteolisis.[55, 56] Las proteínas vegetales de transferencia de lípidos no específicas (nsLTP), altamente resistentes a la acción enzimática, también forman una estructura compacta en forma de barril, constituido por 4 hélices alfa estabilizadas por 4 puentes disulfuro.[57]

La estructura cuaternaria puede ser un aspecto estructural importante, dificultando la accesibilidad de las enzimas proteolíticas a los epítopos de la proteína. Tal es el caso del

alérgeno del cacahuete *Ara h 1*, que forma homotrímeros y, junto con su estructura globular compacta, resulta especialmente estable al ataque por proteasas.[58]

Muchas proteínas extracelulares (incluyendo muchos alérgenos alimentarios) sufren glicosilaciones a su paso por el retículo endoplasmático. Se ha descrito la existencia de IgE específica frente a carbohidratos del alérgeno del tomate *Lyc e 2*, capaz de inducir la liberación de histamina por basófilos.[59] Asimismo, en el alérgeno del huevo ovomucoide, altamente glicosilado, los carbohidratos aportan una estabilidad adicional frente a la desnaturalización térmica e hidrólisis por tripsina.[60]

Un número considerable de alérgenos tienen la capacidad de unir ligandos, desde iones metálicos a lípidos. Esta propiedad funcional puede reducir la movilidad del esqueleto polipeptídico, aumentando tanto la estabilidad térmica como la resistencia a la proteolisis, ya que muchas enzimas digestivas requieren sustratos flexibles. Proteínas como la β-lactoglobulina (βLg) o la proteína de transferencia de lípidos del maíz, poseen un bolsillo hidrofóbico para la unión de lípidos, aumentando su estabilidad térmica al hallarse ocupado.[61, 62] Otras proteínas, como las parvalbúminas del músculo de muchos peces, sufren un cambio conformacional importante al perder el calcio unido a dos dominios específicos de la proteína, de modo que desaparecen varios epítopos de unión a IgE.[63]

1.2.4. Influencia de la matriz y el procesado

Los alérgenos alimentarios se encuentran inmersos en una matriz de diversos compuestos, tales como lípidos, carbohidratos u otras proteínas, formando estructuras complejas dentro del alimento. Si a esto sumamos que muchos alimentos son procesados tecnológicamente (calentamientos, altas presiones, glicaciones, etc.), resulta que los constituyentes individuales se reestructuran en forma de dispersiones coloidales, emulsiones, fases amorfas o cristalinas, o geles inducidos por el calor, el enfriamiento y la aplicación de fuerzas de cizalla. Por tanto, las propiedades físico-químicas de las proteínas pueden verse alteradas significativamente, cambiando la forma en que son degradadas

durante la digestión fisiológica, absorbidas a través del tracto gastrointestinal y/o presentadas al sistema inmunitario del intestino.

Los tipos de modificaciones que sufren las proteínas como consecuencia del procesado comprenden la desnaturalización, la formación de agregados y las modificaciones químicas.

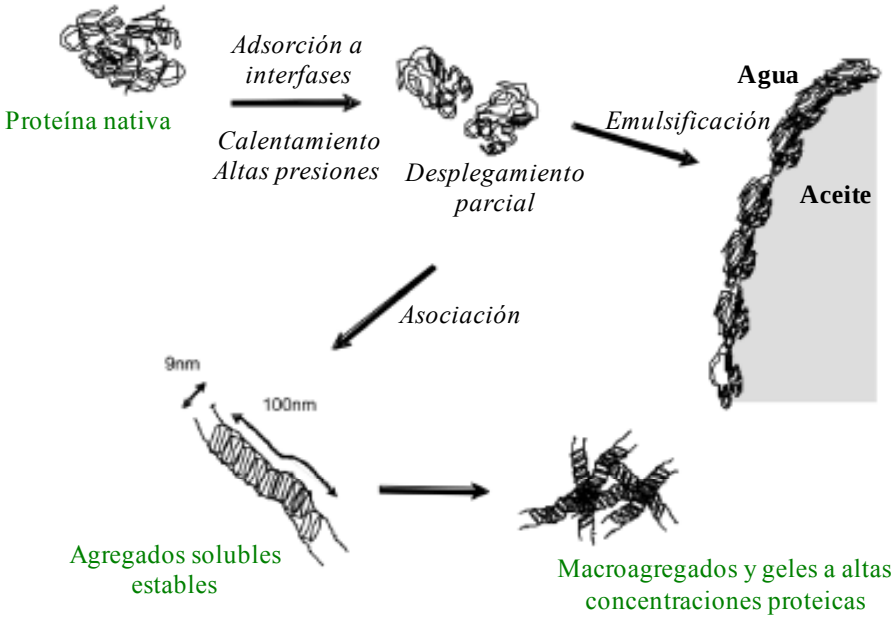


Figura 6. *Mecanismos de desplegamiento y desnaturalización de proteínas como consecuencia del procesado. Tomado de Mills y col.*[64]

La estructura terciaria e incluso secundaria de las proteínas nativas puede verse alterada como consecuencia del calentamiento, el tratamiento con altas presiones, o la adsorción a interfases aire-agua en las espumas, como el merengue o aceite-agua en mayonesas, u otras salsas. El desplegamiento de la proteína puede ir acompañado de cambios en la hidrofobicidad superficial debido a la exposición de residuos que previamente se hallaban ocultos en el interior de la molécula. En consecuencia, y puesto que las proteínas se encuentran a altas concentraciones en muchos alimentos, éstas tienden a formar grandes agregados macromoleculares y redes poliméricas o geles.[65] Factores como la actividad de agua, el pH, la fuerza iónica, la temperatura o la presencia de otros ingredientes, como grasas o azúcares, determinan el patrón y la cinética de

desnaturalización y formación de agregados proteicos. Estas alteraciones en la estructura proteica pueden afectar a la estabilidad frente a las enzimas digestivas.

Muchas proteínas alimentarias se encuentran formando parte de emulsiones. En estos sistemas, las proteínas, en función de su capacidad emulgente, sufren un desplegamiento para exponer sus regiones hidrofóbicas y favorecer las interacciones con los lípidos en la interfase aceite-agua. Se ha descrito, por ejemplo, que la adsorción de los alérgenos de la leche βLg y β-caseína a la interfase aceite-agua altera su susceptibilidad a la digestión gastrointestinal,[66] aunque es importante también el papel que juegan los surfactantes naturales, como las sales biliares, en este modelo. Por otra parte, el alérgeno α-lactalbúmina (αLa) adopta una estructura desplegada de *molten globule* a pH ácido que le permite, merced al aumento de hidrofobicidad superficial, insertarse en las vesículas de fosfatidilcolina, un fosfolípido abundante en la leche, quedando protegido parcialmente de la digestión por pepsina.[67]

Los alérgenos homólogos al alérgeno del polen *Bet v 1*, como *Pru av 1* de la cereza, parecen presentar únicamente epítopos conformacionales.[68] En este caso, el calentamiento produce la desnaturalización y pérdida de dichos epítopos y, en consecuencia, reduce la capacidad de producir reacciones alérgicas en individuos sensibilizados. Por otro lado, existen alérgenos cuya alergenicidad no se ve alterada por el tratamiento térmico. Las caseínas o las prolaminas del trigo poseen una estructura no globular, denominada reomórfica, caracterizada por distintas estructuras secundarias en equilibrio unas con otras, de forma que con el calentamiento no presentan la transición cooperativa entre el estado nativo y desplegado típicamente observada en proteínas globulares más ordenadas. Esta propiedad las hace poseedoras de muchos epítopos lineales termoestables, razón por la cual su capacidad de unión a IgE no se ve alterada por el calentamiento.[69, 70]

Además de las alteraciones estructurales inducidas por la desnaturalización o desplegamiento, existen procesos que originan modificaciones químicas en las proteínas alimentarias. La modificación química más común es la glicación no enzimática por reacción de Maillard. En presencia de azúcares no reductores, y a temperatura y actividad de agua apropiadas, los grupos amino libres de las proteínas reaccionan con los grupos aldehído o

cetona de los azúcares originando un amplio rango de aductos denominados compuestos de Amadori. La reacción de Maillard puede afectar a la alergenicidad de las proteínas alimentarias. Así por ejemplo, los alérgenos del cacahuete *Ara h 1* y *Ara h 2*, glicados por reacción de Maillard, reaccionan entre ellos formando agregados de alto peso molecular que son más resistentes a la digestión gástrica y unen más eficazmente IgE del suero de pacientes alérgicos.[71] Igualmente, el alérgeno de la leche βLg glicado con distintos carbohidratos también presenta una menor degradación enzimática y por tanto conserva mayor capacidad de unir IgE.[72] Las proteínas del huevo ovalbúmina y ovomucoide son otro ejemplo de la influencia de la glicación en la digestibilidad e inmunorreactividad de los alérgenos alimentarios.[73]

1.3. La alergia al huevo

Entre un 0.5 y un 2.5 % de la población presenta alergia al huevo[7]. La edad media de aparición de la enfermedad se sitúa entorno a los 10 meses, coincidiendo en muchos casos con la introducción de la clara en la dieta por primera vez. En raras ocasiones, se producen reacciones adversas sin ingestión previa del alimento, lo que podría achacarse a la sensibilización vía uterina o a través de la leche materna. En adultos, se han descrito casos de sensibilización a través de las vías respiratorias, consecuencia de la inhalación de ovoproductos en polvo por trabajadores de la industria panadera o confitera.

Las reacciones alérgicas al huevo son principalmente cutáneas y aparecen normalmente en los primeros 30 minutos tras la toma o contacto con el alimento. Existe una importante asociación entre la alergia al huevo y la dermatitis atópica. Dos tercios de los casos de dermatitis atópica ocurren en niños y adolescentes alérgicos al huevo,[74] que además cursan una dermatitis más grave y persistente. También son frecuentes los síntomas respiratorios (asma, rinoconjuntivitis, etc.) o gastrointestinales (diarrea, vómitos, dolor abdominal, etc.). Raramente se producen reacciones anafilácticas al huevo, siendo éste responsable de menos del 10% de los casos de anafilaxia en niños.[75]

El tratamiento de la alergia al huevo se fundamenta en una dieta estricta libre de alimentos que puedan contener huevo. En la infancia se lleva a cabo un seguimiento del crecimiento para asegurar que no exista una deficiencia nutricional. Tanto los padres como los niños reciben un plan de actuación de emergencia en caso de ingestión accidental y a menudo se suministra un autoinyector de adrenalina a aquellos pacientes con riesgo de anafilaxia.

1.3.1. Tolerancia al huevo e inmunoterapia oral

El desarrollo de tolerancia con la edad es un fenómeno común, tanto que alrededor de la mitad de los niños alérgicos superan el desorden a los 10 años, y entre el 80 y el 95% tras cumplir los 18 años de edad.[76]

Numerosos estudios han confirmado la mayor tasa de tolerancia de ovoproductos calentados en pacientes alérgicos al huevo.[77, 78] Este hecho se atribuye al efecto del calentamiento sobre la estructura terciaria de las proteínas. Así, los epítopos conformacionales, que dependen de dicha estructura, pueden perderse como consecuencia de la desnaturalización inducida por calor. En dicha circunstancia, el reconocimiento del alérgeno por los anticuerpos IgE específicos solo tendría lugar hacia los epítopos lineales, que únicamente dependen de la secuencia primaria de la proteína. Un estudio de Jarvinen y col.[79] mostró que los pacientes con alergia persistente al huevo reconocían un mayor número de epítopos lineales en el ovomucoide que los pacientes que desarrollaron tolerancia. Otros autores han sugerido que el consumo regular de huevo tratado térmicamente puede influir decisivamente en el curso natural de la enfermedad, favoreciendo la aparición de tolerancia.[80] Adicionalmente a la pérdida de epítopos conformacionales, existen otras explicaciones posibles a la mayor tolerancia de alimentos calentados. En el caso de los alérgenos de la leche, por ejemplo, el tratamiento térmico produce la agregación de αLa y βLg, impidiendo su absorción a través de los enterocitos intestinales.[81]

A pesar del frecuente desarrollo de tolerancia con la edad y la baja incidencia de alergia al huevo calentado, los casos de individuos que presentan reacciones adversas al

huevo ocupan el segundo puesto entre los episodios clínicos de alergia. Estos pacientes han de someterse a dietas restrictivas que presentan varios inconvenientes. Por un lado, son únicamente preventivas y difíciles de cumplir debido a los posibles consumos accidentales, no tienen ningún efecto sobre el curso natural de la enfermedad y, además, pueden conllevar deficiencias nutricionales en niños y rebajar los niveles de alérgeno necesarios para producir síntomas clínicos, agravando así las consecuencias de ingestiones inadvertidas. Por ello, se han intentado múltiples estrategias terapéuticas, principalmente la inmunoterapia oral. Pese a que actualmente se desaconseja su práctica de rutina en la clínica debido a la alta incidencia de reacciones adversas,[82] varios investigadores han logrado distintos grados de éxito. Así, Patriarca y col.[32] llevaron a cabo un protocolo estandarizado de inmunoterapia oral en 13 pacientes alérgicos al huevo de edades comprendidas entre 3 y 55 años. Once de ellos se desensibilizaron tras un periodo de 3-8 meses. Posteriormente, Buchanan y col.[83] administraron un protocolo de inmunoterapia oral durante 24 meses a 7 niños alérgicos, 4 de los cuales acabaron tolerando 10g de huevo en la prueba de provocación, y los otros 3 aumentaron significativamente su límite de tolerancia. Si bien estas terapias solo inducen una desensibilización temporal, producen beneficios evidentes al aumentar en muchos casos la dosis necesaria para sufrir una reacción alérgica, y están especialmente aconsejadas en pacientes con alergia persistente, que no han desarrollado tolerancia con la edad.

1.3.2. Los alérgenos del huevo

Los principales alérgenos del huevo se encuentran fundamentalmente en la clara y son proteínas muy abundantes en ésta: ovomucoide (*Gal d 1*), ovalbúmina (*Gal d 2*), lisozima (*Gal d 3*) y ovotransferrina (*Gal d 4*). Hasta la fecha también han sido descritos dos alérgenos en la yema: α-livetina (*Gal d 5*) y la proteína YGP-42 (*Gal d 6*).

1.3.2.1 Ovomucoide

El Ovomucoide (OM o *Gal d 1*) es una glicoproteína de 28 kDa y punto isoeléctrico 4.1 que representa el 11% (p/p) de las proteínas de la clara. Su secuencia está constituida por 186 aminoácidos y posee 9 puentes disulfuro intramoleculares y aproximadamente un

25% de carbohidratos. La molécula tiene actividad inhibidora de tripsina y está formada por tres dominios homólogos en tándem. Diversos autores han estudiado los epítopos reconocidos por IgE mediante ensayos de unión del suero de pacientes alérgicos a péptidos derivados de la proteína.[84] De esta forma, se han descrito diferentes epítopos en los tres dominios del alérgeno, siendo los epítopos lineales del tercer dominio los inmunodominantes.[85] También se ha señalado que los epítopos de unión a IgE contienen preferentemente residuos hidrofóbicos críticos para la interacción con dicha inmunoglobulina.[86] Por otro lado, obteniendo linfocitos T de pacientes alérgicos al huevo, Holen y col.[87] caracterizaron 10 epítopos de unión al receptor de la célula T, de los cuales 6 también eran reconocidos por la IgE del suero. En la figura 7 se representan los epítopos IgE y TCR encontrados por distintos autores.

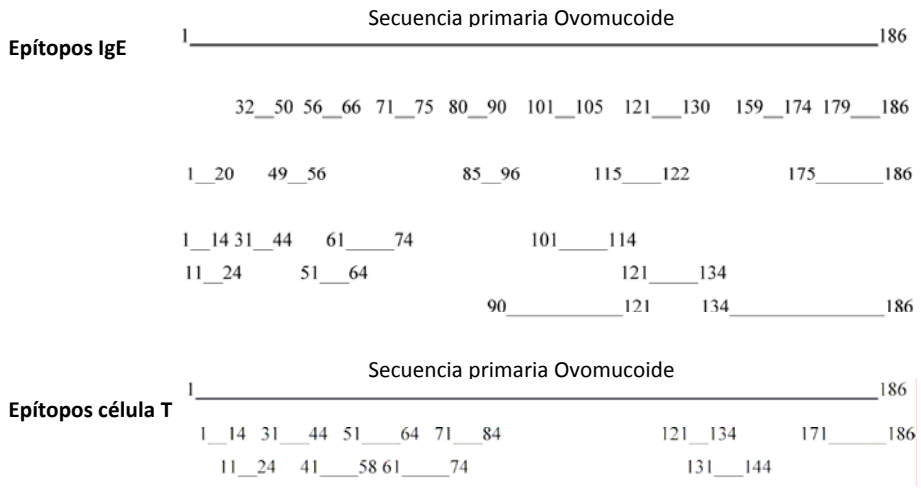


Figura 7. *Representación esquemática de los epítopos del ovomucoide reconocidos por IgE y por la célula T, descritos por distintos autores. Tomado de Mine y Yang.*[88]

El ovomucoide se caracteriza por su alta estabilidad térmica y resistencia a otras formas de desnaturalización, propiedad que se atribuye a la presencia de los 9 puentes disulfuro de su molécula. De hecho, la reducción de los enlaces disulfuro conlleva un aumento de su digestibilidad por pepsina y una reducción de su alergenicidad.[89] El tratamiento con enzimas digestivas en condiciones fisiológicas también produce una disminución de la capacidad de unión a IgE, aunque ésta persiste incluso cuando la proteína ha sido pretratada térmicamente o glicada por reacción de Maillard.[73]

1.3.2.2 Ovalbúmina

La ovalbúmina (OVA o *Gal d 2*) es la más abundante de las proteínas del huevo (54% de las proteínas de la clara) y la más alergénica junto al ovomucoide. Es una fosfoglicoproteína de 45 kDa perteneciente a la superfamilia de las serpinas, pero que a diferencia de éstas no tiene actividad inhibidora de proteasas. Su secuencia tiene 385 aminoácidos y prácticamente toda su cadena polipeptídica presenta motivos de estructura secundaria definidos (hélice alfa y lámina beta). Presenta un puente disulfuro accesible al disolvente y cuatro grupos sulfhidrilo libres en el interior de la molécula.

En la figura 8 se muestran los epítopos de unión a IgE y célula T encontrados por distintos grupos. La composición fisicoquímica de las regiones alergénicas es mayoritariamente hidrofóbica, siendo este tipo de residuos y también los cargados, críticos en la unión a IgE. Las principales estructuras secundarias que contienen los epítopos son láminas beta y giros beta.

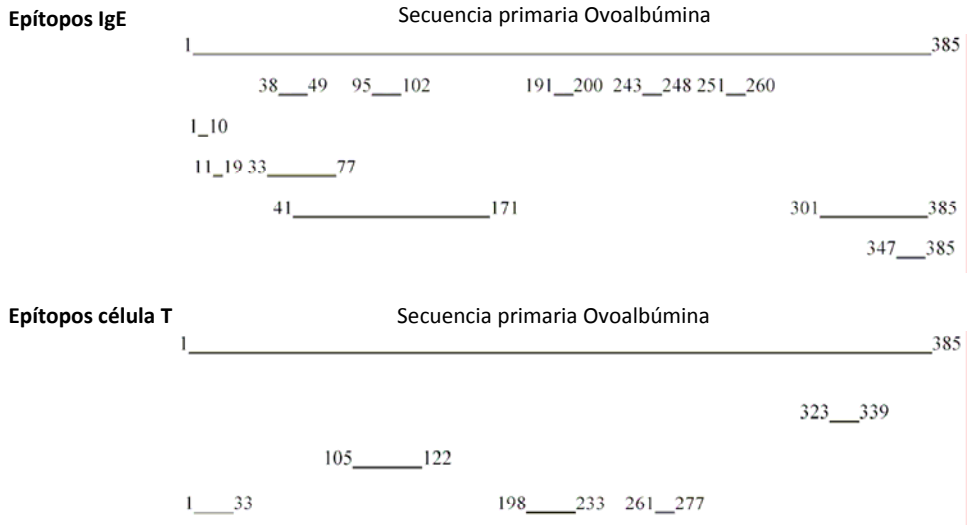


Figura 8. *Representación esquemática de los epítopos de la ovalbúmina reconocidos por IgE y por la célula T, descritos por distintos autores. Tomado de Mine y Yang.*[88]

La OVA se ha revelado resistente a la digestión por pepsina a pH 1.2 durante más de 60 minutos.[1] Otro estudio ha mostrado que el alérgeno es bastante estable en fluido

gástrico simulado y en fluido intestinal simulado.[90] Por otro lado, la OVA es una proteína lábil al calor, que induce su desnaturalización. Varios estudios han demostrado una disminución del reconocimiento de IgE cuando la proteína es calentada a temperaturas superiores a 90 ^{o}C.[91] Además, el tratamiento térmico aumenta su susceptibilidad a la proteolisis y su capacidad final de unión a IgE.[73]

1.3.2.3 Ovotransferrina

La ovotransferrina (OVT o *Gal d 3*) es una glicoproteína de 686 aminoácidos, con masa molecular de 77 kDa y punto isoeléctrico entre 5.6 y 6.2. Su estructura es monomérica, con 15 puentes disulfuro. Presenta una abundancia del 12% de las proteínas de la clara, siendo ésta la concentración más alta de cualquier transferrina encontrada *in vivo*. Tiene actividad transportadora de hierro y bacteriostática gracias a su capacidad de unir dicho metal. También se le han atribuido efectos inmunomoduladores y antioxidantes.

Aún no se han caracterizado los epítopos responsables de la alergia aunque se ha documentado la existencia de reactividad cruzada entre la OVT y componentes de la yema del huevo.[92] Presenta epítopos tanto lineales como conformacionales y es lábil al calor, ya que su antigenicidad se ve reducida con el tratamiento térmico a 95 ^{o}C durante 15 minutos.[91]

1.3.2.4 Lisozima

La lisozima (LYS o *Gal d 4*) es también una glicoproteína, de masa molecular 14.3 kDa y punto isoeléctrico 11. Constituye únicamente un 3.4% del contenido proteico total de la clara de huevo. Presenta cuatro enlaces disulfuro en su molécula y es bien conocida por su actividad bacteriolítica frente a organismos procariotas.

Hasta la fecha, no se han descrito los epítopos de unión a IgE o a la célula T, aunque un estudio identificó respuestas T-específicas frente a lisozima en células mononucleares de sangre periférica (PBMCs) de pacientes alérgicos al huevo.[93]

La LYS es estable en el rango de pH 3.5 a 5, presentando una temperatura de desnaturalización entorno a 80 ^{o}C. A pHs inferiores su estabilidad disminuye rápidamente.

Se ha descrito que el alérgeno es resistente a la digestión por pepsina y proteinasa K a 37 ^{o}C durante 60 minutos.[94]

1.3.2.5 Alérgenos de la yema

Si bien en un principio se pensaba que la yema estaba libre de componentes alergénicos, han sido numerosos los estudios que describen reactividad de la yema frente a IgE de pacientes alérgicos. El alérgeno de la yema más veces referido es la α-livetina (*Gal d 5*) o albúmina sérica de pollo. Se trata de una proteína presente en la fracción plasmática soluble de la yema, que produce síntomas alérgicos tras su ingestión por individuos previamente sensibilizados a las plumas de ave (síndrome ave-huevo). Tiene una masa molecular de 66 kDa y presenta 17 puentes disulfuro y un grupo sulfhidrilo libre que puede dar lugar a dímeros. Constituye menos del 10% de las proteínas de la yema y se ha descrito reactividad cruzada con la OVT. Es un alérgeno lábil térmicamente, pues su capacidad de unión a IgE se ve reducida más de un 80% al calentarlo a 90 ^{o}C durante 30 minutos.[95]

Recientemente se ha identificado otro alérgeno presente en el plasma de la yema, la glicoproteína YGP-42 (*Gal d 6*), un fragmento C-terminal de la proteína Vitelogenina I.[4] Se trata de una proteína de 31.4 kDa resistente al calor pero lábil a la digestión por pepsina.

1.4. Digestión gastrointestinal *in vitro*

El cuerpo humano ha desarrollado un sistema complejo de digestión del alimento para obtener de él todos los nutrientes necesarios. En primer lugar, ocurre la masticación en la cavidad bucal, donde el alimento se mezcla con la saliva que contiene la enzima amilasa, que hidroliza glucógeno y almidón. A continuación, tras el paso por el esófago, las proteínas contenidas en el bolo alimenticio llegan al lumen gástrico. Aquí en el estómago, se segrega ácido clorhídrico y proteasas (pepsinas), que pasan a ser activas gracias al pH ácido. Las pepsinas presentan un amplio espectro de especificidad, cortando preferentemente por los residuos de fenilalanina, tirosina y leucina. En el estómago existe fosfatidilcolina, un fosfolípido que, junto al mucus, forma una capa que protege a la mucosa gástrica del pH extremo y las enzimas digestivas.

Después del paso por el estómago, las proteínas y los péptidos remanentes presentes en el quimo son vertidos al duodeno del intestino delgado, donde se exponen a una gran variedad de proteasas producidas y secretadas por el páncreas, tales como tripsina, quimotripsina y carboxipeptidasas, y enzimas de la superficie apical del epitelio intestinal. Estas enzimas requieren un pH más alcalino, propio del intestino, para actuar. También entran en juego las sales biliares, que son sintetizadas en el hígado, concentradas y almacenadas en la vesícula biliar y liberadas en la bilis al duodeno para emulsionar las grasas y facilitar su absorción. En la bilis también hay fosfatidilcolina, sintetizada de forma natural en el hígado. La lipasa y su cofactor colipasa son sintetizadas en el páncreas y secretadas también al duodeno para procesar lípidos de la dieta y convertirlos en compuestos más simples y fácilmente absorbibles por el intestino. Por último, las proteínas y péptidos persistentes son absorbidos por los enterocitos, donde podrán sufrir un último proceso de degradación intracelular antes de alcanzar la lámina propia.

Aunque clásicamente se pensaba que solo di- o tripéptidos podían llegar a la submucosa intestinal, hoy en día se conoce que pueden penetrar a la capa serosa péptidos inmunológicamente activos vía enterocitos, células dendríticas, células M en las placas de Peyer y/o difusión paracelular.[96] Así, aunque la mayoría de las proteínas son absorbidas como péptidos o completamente degradadas en aminoácidos, una pequeña porción puede atravesar intacta el epitelio e incluso alcanzar la circulación sanguínea. Se ha podido detectar, por ejemplo, la βLg bovina a una concentración entre 0.1 y 3 ng/mL en sangre a partir de los 30 minutos del consumo de 1.2 L de leche de vaca.[97] También para la OVA se han descrito cantidades de hasta 10 ng/mL en suero 2 horas después de la ingestión de huevo.[98] La cantidad de proteína intacta que llega a la circulación puede incluso verse aumentada bajo ciertas condiciones de enfermedad o en el período perinatal con un sistema digestivo inmaduro.

Ya se ha mencionado que la estabilidad estructural es una característica muy extendida entre los alérgenos alimentarios. Esta propiedad asegura que el alérgeno sobrevive al paso por el sistema digestivo conservando integridad estructural suficiente como para ser absorbido por el intestino y sensibilizar al sistema inmunitario de la mucosa.

La alta estabilidad también permite que una forma inmunológicamente activa del alérgeno pueda alcanzar a las células efectoras y desencadenar, por tanto, la respuesta alérgica en individuos sensibilizados.

Con objeto de entender la fase de provocación de la respuesta alérgica, es importante estudiar las proteínas nativas y los fragmentos (y agregados) producidos durante la digestión para evaluar si mantienen su alergenicidad durante el tránsito gastrointestinal. En 1996, Astwood y col.[1] fueron capaces de distinguir proteínas alergénicas de no alergénicas mediante un estudio de digestión empleando fluido gástrico simulado (SGF) que contenía la enzima pepsina. Otros autores observaron que la digestión en SGF de varios alérgenos del pescado reducía la capacidad de unión a IgE hasta 10000 veces y la liberación de histamina.[99]

Sin embargo, estudios posteriores han arrojado dudas acerca del uso de la resistencia a la pepsina como criterio para predecir la alergenicidad, ya que se ha visto que muchas proteínas alergénicas no son más resistentes que otras no alergénicas.[3, 100] La estabilidad aparente de una proteína puede variar dependiendo de las condiciones experimentales utilizadas (pH, relación pepsina-proteína, pureza y métodos de detección). De hecho, la susceptibilidad a la digestión de un mismo alérgeno puede variar sustancialmente de unos estudios a otros. Además, la consideración de componentes de la matriz del alimento en el modelo de digestión puede cambiar drásticamente el resultado de la misma. Por ello, se han desarrollado modelos más complejos que la pepsinolisis, generalmente multifásicos, que tratan de considerar muchos de los factores relevantes en la digestión fisiológica.

1.4.1. Modelos de digestión fisiológicamente relevantes

La adquisición de datos acerca de la composición de los fluidos presentes a lo largo del tracto gastrointestinal humano, tanto en situación pre- como postprandial, ha servido de base para el desarrollo de modelos *in vitro* que recreen las condiciones encontradas *in vivo*. Los modelos estáticos bifásicos son la elección más frecuente, ya que permiten un fácil seguimiento del proceso digestivo y reproducen la composición diferencial que existe en los

medios estomacal e intestinal (Fig. 9). Este tipo de modelos se usan preferentemente para el estudio de alimentos simples o nutrientes aislados, así como de proteínas alergénicas concretas. En rasgos generales, incluyen la homogenización del alimento, acidificación con ácido clorhídrico, adición de enzimas gástricas seguida de incubación durante un periodo variable de residencia simulada en el estómago, neutralización con bicarbonato o hidróxido sódico y adición de enzimas pancreáticas y sales biliares mientras se agita a 37 ^{o}C. Se emplea la tasa de desaparición de un componente o la de aparición de otro como medida de la progresión de las reacciones digestivas.

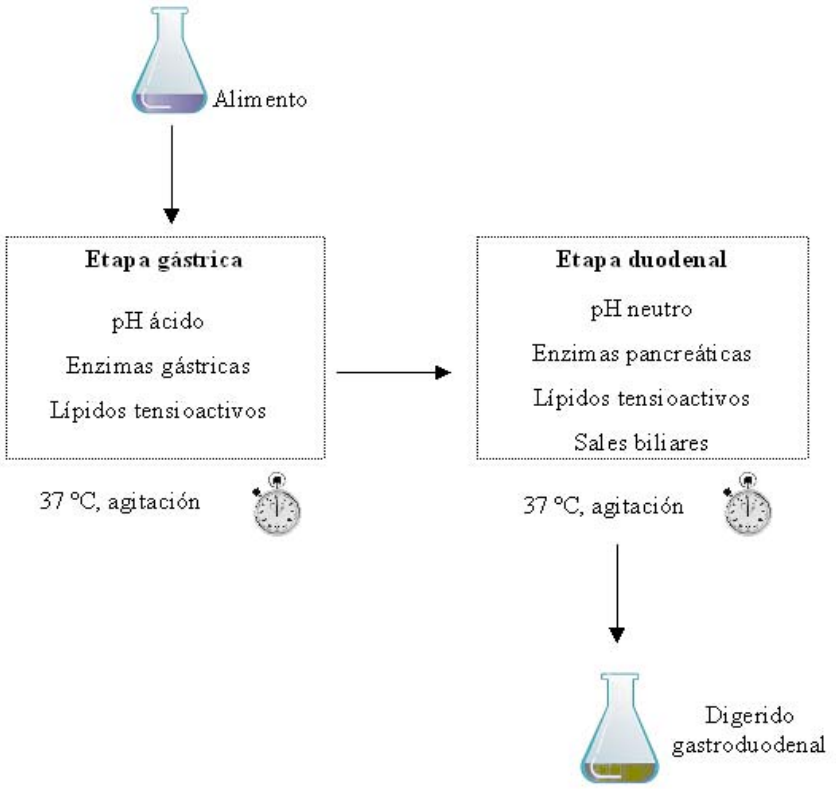


Figura 9. *Representación esquemática de un modelo bifásico de digestión gastroduodenal.*

La hidrólisis de proteínas es despreciable en la boca pero adquiere gran relevancia en el estómago y en el intestino. Estas dos regiones difieren respecto al pH, la concentración de surfactantes y la cantidad y tipo de enzimas, por lo que son simuladas de forma secuencial en los modelos de digestión. No obstante, la variabilidad de estos parámetros entre los modelos utilizados por distintos autores origina a menudo resultados de digestibilidad contradictorios. Por ejemplo, Fu y col.[100] observaron que a pH 1.2 y una

relación pepsina/proteína de 13 (p/p), los alérgenos OVA y OM se degradaban en 5 y 0 min, respectivamente, mientras que otros autores describieron una estabilidad de 60 min de la OVA cuando la relación enzima/ sustrato bajaba a 3 (p/p)[101] o de 8 min en el caso del OM cuando la relación era de 19 (p/p).[1] Por tanto, se hace necesario establecer unas condiciones que se asemejen lo más posible a las encontradas fisiológicamente, de modo que los resultados de digestibilidad reflejen lo que ocurre *in vivo*.

En el caso de la pepsina, no es fácil elegir una relación pepsina/proteína óptima puesto que varía enormemente dependiendo del individuo y el menú ingerido.[102] No obstante, se estima que la concentración de proteína generalmente excede a la de enzima, por lo que las relaciones enzima/alérgeno usadas tradicionalmente en los protocolos de digestión *in vitro* serían órdenes de magnitud superiores a las halladas *in vivo*.[103] Además, se ha descrito que altas concentraciones de NaCl incrementan la actividad enzimática de la pepsina y su espectro de especificidad,[104] por lo que es conveniente emplear una concentración fisiológica de esta sal, que se ha sugerido estar entorno a 35 mM.[105]

Como ya se ha comentado, el pH estomacal también puede influir decisivamente en la digestibilidad de las proteínas de la dieta. Por ejemplo, la proteína de la leche αLa, que es digerida rápidamente por pepsina a pH 2, se torna muy resistente a pH 4.[106] El pH gástrico en individuos sanos se encuentra por debajo de 3 en condiciones de ayuno, con un rango intercuartílico de pH de 1.4 a 2.1. Por tanto, un medio de disolución adecuado para simular las condiciones gástricas preprandiales debe tener un pH entre 1.5 y 2.[105] Este valor aumenta tras la ingesta dependiendo de la composición y tamaño del menú ingerido, pero vuelve progresivamente a valores más ácidos. En el intestino, la secreción de bicarbonato por el páncreas neutraliza el ácido procedente del estómago, de forma que los valores de pH duodenal oscilan entorno a 6.5.

Se ha demostrado que la presencia, en concentraciones fisiológicas, de surfactantes en los medios de digestión, puede afectar a la digestibilidad de los alérgenos. La fosfatidilcolina es un componente lipídico fundamental de la mucosa gástrica y puede ser sintetizado por ésta bajo ciertas condiciones. También está presente en diversos alimentos,

como en el huevo o la leche. Moreno y col.[67] emplearon un modelo de digestión *in vitro* que incluía cantidades fisiológicas de este fosfolípido para el estudio del alérgeno de la leche αLa, observando que dicha proteína era capaz de insertarse en las vesículas del fosfolípido, quedando menos accesible para la acción enzimática. Otros autores también mostraron que la fosfatidilcolina protegía de la degradación a la βLg.[66] Por otro lado, las sales biliares secretadas al duodeno pueden desorber de la interfase proteínas emulsionadas,[107] afectando a su concentración en solución y su disponibilidad para el ataque enzimático. Por tanto, la inclusión de ambos surfactantes en los modelos de digestión se hace necesaria para la adecuada recreación de lo que sucede *in vivo*.

La presencia de fases lipídicas en el medio de digestión puede tener un efecto crucial en la degradación enzimática de alérgenos y ha sido considerada en diversos estudios. Burnett y col.[108] estudiaron el comportamiento de una serie de alérgenos proteicos en emulsiones modelo bajo condiciones gástricas y duodenales. Varios alérgenos mayoritarios eran capaces de adsorberse a emulsiones gástricas y después desorberse bajo condiciones duodenales. Dado que las proteínas adsorbidas a una emulsión pueden ser menos susceptibles a la pepsinolisis que las que se encuentran libres en solución, este fenómeno facilitaría la llegada de alérgenos emulsionados al intestino, donde las condiciones del medio, tales como la presencia de sales biliares, promoverían su liberación. Además, experimentos de espectroscopía de fluorescencia han demostrado que las proteínas adsorbidas a interfases aceite:agua se pueden encontrar parcialmente desnaturalizadas y hallarse, en cierta medida, apartadas del entorno acuoso y menos disponibles para la hidrólisis enzimática.[109]

Si se examinan los efluentes del íleo humano tras la ingesta, se aprecia que el alimento se encuentra lejos de haber sido digerido completamente. A menudo es posible identificar qué es lo que esa persona ha comido. El grado de absorción de un nutriente, la velocidad y el lugar del tracto gastrointestinal donde esto ocurre, no solo depende del ambiente luminal, sino también de las características físicas del alimento. Estos hechos cobran especial importancia en alimentos estructurados más complejos, para los que es posible el empleo de modelos dinámicos, que incorporan las fuerzas mecánicas y los cambios temporales de las condiciones luminales que tienen lugar *in vivo*.[110]

1.4.2. Modelos de absorción intestinal

La superficie de la mucosa del tracto intestinal contiene una única capa de células epiteliales. La mayor parte del epitelio consiste en enterocitos alineados que sellan la entrada paracelular de posibles proteínas y péptidos antigénicos mediante uniones íntimas. El transporte de macromoléculas a través de estas uniones es, por tanto, poco frecuente, siendo preferente la vía transcelular. Dicha ruta es mayoritariamente degradativa, aunque se ha descrito también el transporte de proteínas intactas.[111]

Diversos estudios *in vivo* han demostrado la absorción gastrointestinal de varios alérgenos alimentarios, tales como la albúmina sérica bovina,[112] ovalbúmina,[113] *Gly m Bd 30k* de la soja[114] y proteínas del cacahuete.[96] Sin embargo, los estudios *in vivo* conllevan la dificultad de tener que suministrar altas dosis de alérgeno purificado para evaluar su transporte y tasa de absorción. Por este motivo, a menudo se recurre a modelos *in vitro* empleando líneas celulares que se asemejan al epitelio intestinal humano. Uno de los modelos más ampliamente utilizados son las células de adenocarcinoma de colon humano, *Caco-2*. Esta línea celular, a pesar de provenir del colon, sufre un proceso espontáneo de diferenciación en cultivo hacia células columnares polarizadas análogas a los enterocitos del intestino (Fig. 10). En concreto, presentan microvellosidades bien desarrolladas[115] y excretan enzimas del borde del cepillo intestinal.[116] En los ensayos de transporte, las células *Caco-2* se cultivan en filtros porosos, donde su diferenciación conduce a la formación de una monocapa funcionalmente polarizada de células que separa dos compartimentos extracelulares muy distintos: el apical y el basolateral. La información de permeabilidad obtenida de este modelo presenta una buena correlación con la absorción intestinal en humanos.[117]

Sin embargo, el modelo de células *Caco-2* tiene varias limitaciones derivadas de su origen colónico. Por un lado, forman monocapas altamente compactas con baja permeabilidad paracelular para compuestos hidrofílicos y presentan baja absorción de compuestos que requieren transporte transcelular activo mediado por receptor.[118] Además, las monocapas solo están compuestas por células columnares absortivas (enterocitos), mientras que el intestino humano presenta un conglomerado de células en el que además

de enterocitos hay células enteroendocrinas, células de Paneth, células M o células caliciformes.

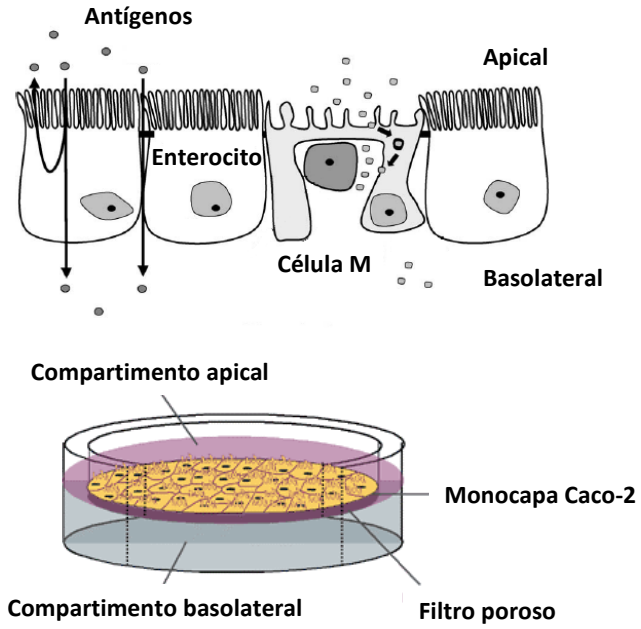


Figura 10. *Representación de las células epiteliales que conforman la barrera intestinal (arriba) y soporte poroso para cultivar las células enterocíticas Caco-2, que crecen formando una monocapa que separa el compartimento apical del basolateral (abajo).*

Las células caliciformes constituyen el 10% de las células del intestino delgado y son, después de los enterocitos, las más abundantes. Tienen una función secretora de mucus, que actúa como una barrera adicional a la entrada de nutrientes o de antígenos. Estas células pueden imitarse *in vitro* empleando líneas celulares secretoras de mucus, como la *HT29-MTX*, una subpoblación de las células *HT29* de adenocarcinoma de colon humano seleccionadas mediante resistencia a metotrexato (MTX). Se han desarrollado modelos de cocultivos de *Caco-2* y *HT29-MTX* que forman monocapas con uniones íntimas entre ambos tipos de células y generan una capa de mucus, asemejándose a las condiciones fisiológicas. Este modelo se ha usado principalmente en estudios de biodisponibilidad de fármacos y nutrientes. Mahler y col.[119] observaron que la absorción de hierro ferroso, en forma hemo o formando parte de la ferritina era menor en cocultivos que en cultivos puros de células

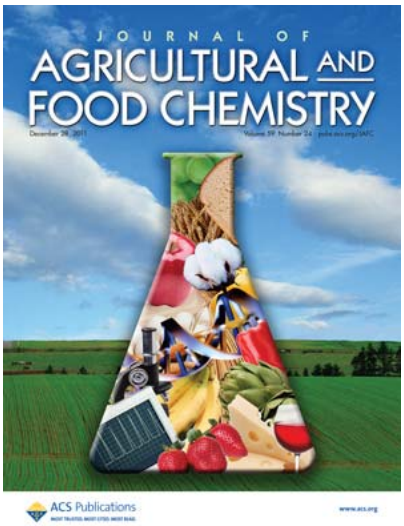
Caco-2. También se ha demostrado un transporte paracelular aumentado de compuestos hidrofílicos en cocultivos *Caco-2/HT29-MTX* sembrados en proporción fisiológica 1:9.[118]

Las células *Caco-2* también han sido empleadas en cocultivos de doble capa junto a células *Hep G2* para investigar la toxicidad de algunos compuestos. En dicho modelo, las células *Caco-2* son sembradas en filtros porosos de forma análoga a como se muestra en la Fig. 10. Estos filtros se sitúan en pocillos donde además existe una capa basal de células *Hep G2*, destinadas a imitar los procesos metabólicos hepáticos que suceden al transporte intestinal. Así, Choi y col.[120] obtuvieron una buena correlación entre la toxicidad *in vivo* del tóxico benzo-α-pireno y la viabilidad de las células *Hep G2* en el cocultivo.

Otro tipo fundamental de células epiteliales son las células M que recubren las placas de Peyer del intestino. A través de estas células, los antígenos pueden acceder mediante transcitosis a los folículos linfoides de la mucosa. A diferencia de los enterocitos, en las células M la ruta mayoritaria de transporte es la endocitosis no degradativa, proporcionando antígenos intactos al tejido linfoide.[121] Algunos estudios han obtenido este tipo de células provocando la diferenciación de células *Caco-2* mediante cultivo conjunto con linfocitos procedentes de las placas de Peyer de ratón y han usado dicho modelo para evaluar el transporte de alérgenos como ovalbúmina[122] o βLg.[123] Se demostró que la ovalbúmina era transportada por las células M y era capaz sucesivamente de activar a las células T específicas. En el caso de la βLg, su transporte fue mayor a través de células M que en cultivos *Caco-2*, y se observó que el tratamiento térmico dificultaba la absorción de la proteína.

2. RESULTADOS

I



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ARTICLE

Egg White Ovalbumin Digestion Mimicking Physiological Conditions

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Gastrointestinal digestion of ovalbumin (OVA) was simulated using an in vitro system in two steps, which mimicked digestion in the stomach and duodenum, to assess the effect of different gastric pHs, different concentrations of proteases, and the presence of surfactants, such as phosphatidylcholine (PC) and bile salts (BS). OVA was very resistant to pepsin action at an enzyme/substrate ratio that would resemble a physiological situation (1:20 w/w, 172 units/mg) at pH values equal or above 2. The presence of PC did not change the susceptibility of OVA to proteolysis with pepsin. Fluorescence experiments showed that OVA interacted with PC vesicles, particularly at acidic pH, but it is likely that the protein maintained a high degree of conformational stability, resisting pepsin action. The presence of BS at physiological concentrations considerably increased the proteolysis of OVA by a mixture of pancreatic enzymes. The addition of PC made OVA even more sensitive to proteolytic degradation, suggesting that OVA could associate with the surfactants under duodenal conditions, increasing its exposure to pancreatic proteinases. Immunoreactivity against IgE from sera of allergic patients was retained after in vitro gastric digestion, depending on the reactivity of the sera, but it decreased considerably after in vitro duodenal digestion.

KEYWORDS: Ovalbumin; in vitro digestibility; pepsin; pancreatic enzymes; physiological surfactants; IgE-binding

INTRODUCTION

Egg represents, together with cow's milk, the most common cause of allergic reactions to food that affect approximately 6% of children and 3−4% of adults in the U.S., and ovalbumin (OVA, Gal d 2), the major protein in egg white (58% w/w), is considered a dominant allergen (*1*). OVA is a glycoprotein with a molecular mass of 45 kDa. Its sequence comprises 385 amino acids and includes six cysteines with a single disulfide bond between Cys_{73} and Cys_{120}. OVA partially resists hydrolysis with pepsin, a characteristic that is also shared by many allergenic proteins, which withstand processing and digestion in the gastrointestinal tract, mediated by proteolytic enzymes, low pH, and surfactants such as phospholipids and BS, and thus keep a certain degree of three-dimensional structural integrity to trigger the immune reaction (*2*). In fact, stability to digestion is used as a criterion to evaluate the allergenic potential of novel proteins, such as transgenic proteins.

While a general agreement on the proteolytic stability of many food allergens exists, a lack of correlation between in vitro digestibility and allergenicity has been reported by many authors (*3*−*5*). This is probably because the digestibility of a protein, as measured by an in vitro assay, is greatly influenced by the conditions used, which commonly imply protease to substrate ratios that are orders of magnitude greater than the ratios found in vivo or ignore the interactions of food proteins with other digestive components. In addition, even in the absence of intact protein, proteolytic fragments generated during digestion may have the potential to bind IgE and elicit an allergic response.

In this work we have simulated the gastrointestinal proteolysis OVA using an in vitro digestion system in two steps, which mimics the successive passage through the stomach and duodenum (*6*, *7*), assessing the effect of different gastric pHs, different concentrations of proteases, and the presence of surfactants, such as phosphatidylcholine (PC) and bile salts (BS). The influence of these processes on the digestibility of OVA and the IgE-binding properties of the resulting digestion products was examined.

MATERIALS AND METHODS

In Vitro Gastric Digestion. OVA (A-2512, grade VI, 99% purity, Sigma, MO, USA) was dissolved in simulated gastric fluid (SGF, 35 mM NaCl) at pH 1.2, 2, and 3.2, preheated for 15 min at 37 °C, and subjected to an in vitro gastric digestion at 37 °C with porcine pepsin (EC 3.4.23.1, 3440 units/mg, Sigma) at an enzyme/substrate ratio of 1:20 w/w (172 units/mg) (*6*, *7*). A highest ratio of 3:1 w/w (10320 units/mg) was also used (*8*). Aliquots were taken at 0, 60, 90, and 120 min, and the reaction was stopped by adding 1 M $NaHCO_3$, giving a final protein concentration of 5 mg/mL and pH 7.

Gastric digestions were also performed at pH 2 and a pepsin/OVA ratio of 1:20 w/w in either the absence or presence of PC (L-α-phosphatidylcholine, P3841; Sigma) as described by Moreno et al. (*6*, *7*). Phospholipid vesicles were prepared by dissolving PC in SGF pH 2 (9.58 mg/mL). To facilitate the homogeneous dispersion, the mix was incubated under agitation at 37 °C and vortexed periodically until complete dissolution. Then, it was sonicated in ice (5 min, raising the power from 10% to 50%,

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and 5 min at 60% power) not exceeding a sample temperature of 40 °C. OVA, dissolved in SGF pH 2, was mixed with the PC vesicle solution (1:1.2 v/v) and preheated, and pepsin was added as already described. In the digestions without PC, the PC solution was replaced by SGF pH 2. After 60 min of incubation, the reaction was stopped by raising the pH to 7 with 1 M $NaHCO_3$, the final protein concentration being 5 mg/mL.

At least triplicate digestions were conducted for each condition.

In Vitro Duodenal Digestion. Duodenal digestions were performed by using, as the starting material, pH 2, 60 min gastric digests (with a pepsin/OVA ratio of 1:20), adjusted to pH 7, as described above, with the addition of 1 M $CaCl_2$, 0.25 M bis-Tris pH 6.5, and a 0.125 M BS mixture containing equimolar quantities of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma) (*6*, *7*). After preheating at 37 °C for 15 min, trypsin (EC 232-650-8, 10100 BAEE units/mg protein, Sigma), α-chymotrypsin (EC 232-671-2; 55 units/mg protein, Sigma), porcine pancreatic lipase (EC 232-619-9, Sigma), and colipase (EC 259-490-1, Sigma) prepared in 35 mM NaCl adjusted to pH 7 were added to the duodenal mix. The final composition of the mixture was 4.15 mg/mL OVA, 6.15 mM of each BS, 20.3 mM bis-Tris, 7.6 mM $CaCl_2$; the enzymes referred to the quantity of protein were 40 units/mg trypsin, 0.5 units/mg α-chymotrypsin, 28.9 units/mg lipase and colipase (enzyme/substrate ratio 1:895 w/w). Duodenal digestions were also performed by replacing trypsin and α-chymotrypsin with a commercial pancreatic enzyme mix, Corolase PP (AB Enzymes GmbH, Darmstadt, Germany) at an enzyme/substrate ratio of 1:25 w/w. To test the influence of BS and lipases, these were replaced, when necessary, with 35 mM NaCl adjusted to pH 7. At least triplicate digestions were conducted for each condition.

SDS-PAGE. Samples were dissolved (1:1 v/v) in 10 mM Tris-HCl buffer, pH 8, containing 2.5% SDS, 5% 2-β-mercaptoethanol, and 10 mM EDTA and heated at 100 °C for 10 min. SDS-PAGE was performed on a PhastSystem Electrophoresis apparatus, using precast Homogeneous Gels 20% and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the electrophoretic and Coomassie Blue and silver nitrate staining conditions of the manufacturer. A LMW Calibration Kit for SDS (Amersham Biosciences) was used.

RP-HPLC. OVA hydrolysates, at a concentration of 2.5 mg/mL, were separated in a Hi-Pore RP-318 (250 mm × 4.6 mm internal diameter) column (Bio-Rad, Richmond, CA, USA), in a Waters 600 HPLC (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and UV detector. The digests were eluted by using 0.37% (v/v) trifluoroacetic acid in double-distilled water as solvent A and 0.27% (v/v) trifluoroacetic acid in acetonitrile as solvent B, at 1 mL/min, and 220 nm (*9*). Data were processed by using Empower 2 Software (Waters Corporation).

Mass Spectrometry Analyses. The protein band of interest was manually excised, and in-gel trypsin digestion was performed with Promega trypsin (Madison, WI, USA) (*10*). MALDI-MS analysis of tryptic peptides was performed on an Ultraflex TOF-TOF Instrument (Bruker Daltonics, GmbH, Bremen, Germany). The matrix material was CHCA on anchor-chip targets (Bruker Daltonics). Identification of the proteins was also carried out by TOF-TOF PSD fragmentation spectra. For ESI-MS/MS, an Esquire HCT (Bruker Daltonics) was used. Database searches were performed using the MASCOT program (Matrix Science).

Fluorescence Spectroscopy. The interaction between OVA, PC, and BS was studied by fluorescence spectroscopy (*11*). Fluorescence spectra between 300 and 380 nm (excitation, 280 nm) were recorded at room temperature on a Shimadzu RF-1501 spectrofluorophotometer. The binding of PC and BS was measured as the change in fluorescence. The following procedure was used for titration of OVA with the phospholipid. The PC vesicle solution was prepared both in SGF pH 2 and in 10 mM phosphate buffer pH 7, at a concentration of 4.1 mg/mL. Two milliliters of 0.15 mg/mL OVA in SGF or in phosphate buffer were placed in a cuvette, and every 5 min, under continuous shaking, 10 μL of the phospholipid solution was added. In order to eliminate the dilution of the OVA solution by the added phospholipid, a blank containing the protein solution, titrated with SGF or phosphate buffer, was monitored as described above. The fluorescence intensity changes of the blank were subtracted from the fluorescence intensity measurements of the OVA-PC mixture for every titration point. In all cases, before correction for the blank, fluorescence intensity of free OVA was normalized to 1. For the titration with BS, 10 μL of 15 mM sodium taurocholate and 15 mM glycodeoxycholic acid was added, either separately or both together.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were measured using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). The far (195−260 nm) and near (250−350 nm) UV CD spectra of OVA, in SGF pH 2 and phosphate buffer 10 mM pH 7, were recorded at 20 °C using cells with respective pathlengths of 0.1 and 0.2 cm. The instrument was calibrated with ammonium D-10-camphorsulfonate. Spectra represent the average of four accumulations collected at 100 nm/min, with a 2 s time constant, a 0.5 nm resolution, and a sensitivity of ±100 mdeg. The samples were dissolved at 0.2 mg/mL for the analysis in the far-UV region and at 2 mg/mL for the near-UV region. The buffer blanks were subtracted from each CD spectrum. PC was dissolved at 0.184 mg/mL or 1.84 mg/mL in either SGF or phosphate buffer, and sodium taurocholate and glycodeoxycholic acid were at 0.29 or 2.9 mM to maintain the ratios used during digestion. The data were expressed as mean residue ellipticity (10^3 deg cm^2/decimol). Empirical determinations of protein secondary structure were obtained employing the CDSSTR algorithm (*12*, *13*) and the reference database SP175 (*14*).

IgG Binding by Direct ELISA. Gastric and duodenal digests, as well as blanks containing the protein in SGF pH 2 or in a buffer containing 20.3 mM bis-Tris methane and 7.6 mM $CaCl_2$, pH 6.5, were properly diluted to a final protein concentration of 2.5 μg/mL and applied in triplicate to microplates (polystyrene microtiter plates, Corning, Cambridge, MA, USA). Each well was coated with 50 μL of the sample and incubated overnight under refrigeration. Afterward, the plate was washed using a Microplate Washer (Nunc, Roskilde, Denmark), and PBS-Tween 20 (PBST, 2.5%) was used as saturating agent to avoid nonspecific binding. After 60 min of blocking, 50 μL of polyclonal rabbit anti-OVA conjugated with horseradish peroxidase (HRP) (DakoCytomation, Glostrup, Denmark), diluted 1:55000 in PBST, was added per well and incubated for 60 min. *o*-Phenylene-diamine (OPD, Palex Medical SA, Sant Cugat del Vallés Barcelona, Spain) was used as substrate, and after 30 min of incubation, the reaction was stopped by the addition of 0.5 M sulphuric acid. Absorbance was measured at 492 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). A negative control without antigen (PBS) and positive controls (with different concentrations of OVA) were included each plate.

Triplicate determinations in two different ELISA plates were carried out, and measurements were averaged. Values are expressed as means ± SD. Significant differences ($P < 0.05$) were evaluated by one-way analysis of variance (ANOVA). PASW (formerly SPSS) was used for data processing (version 14.0, SPSS Inc., Chicago, IL, USA).

Human IgE Binding by Inhibition ELISA. For inhibition ELISA, two individual serum samples from children with clinical allergic symptoms to egg white proteins were used. The sera were collected from the Maternal and Child Gregorio Marañon Hospital (Madrid, Spain). The patients had specific seric IgE levels toward OVA of 25.1 and 78.1 KU/L, as determined by CAP (GE HealthCare, Uppsala, Sweden).

Single wells of polystyrene microtiter plates were coated with 10 μg/mL of OVA solution in 0.01 M PBS, pH 7.4, and incubated overnight at 6 °C. Plates were washed with PBST using the Microplate Washer (Nunc). This washing system was used after each incubation step. Then, serial dilutions of each sample (not less than seven) were incubated during 120 min with patient's sera previously diluted in PBST (1:1 v/v), and 50 μL was added to each well. After 120 min of incubation, 50 μL of HRP-conjugated rabbit antihuman IgE, diluted 1:1000 in PBST, was added per well and incubated for 60 min. The tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Walthman, MA, USA). Finally, OPD was used as substrate, and after 30 min of incubation, the reaction was stopped with 0.5 M sulphuric acid. Absorbance was measured at 492 nm. A negative control without serum (native protein in PBST) and positive controls (sera diluted in PBS) were included in each plate.

A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal curve of inhibition dose-response with variable slope, from which the IC_{50} (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad PRISM 4 for Windows (www.graphpad.com). The IgE binding capacity was expressed as the percentage of the IC_{50} of the intact protein.

Western Blotting. Samples (prepared as described above) were run on a polyacrylamide Tris-HCl (10.5−14% gradient) gel in Tris-glycine-SDS

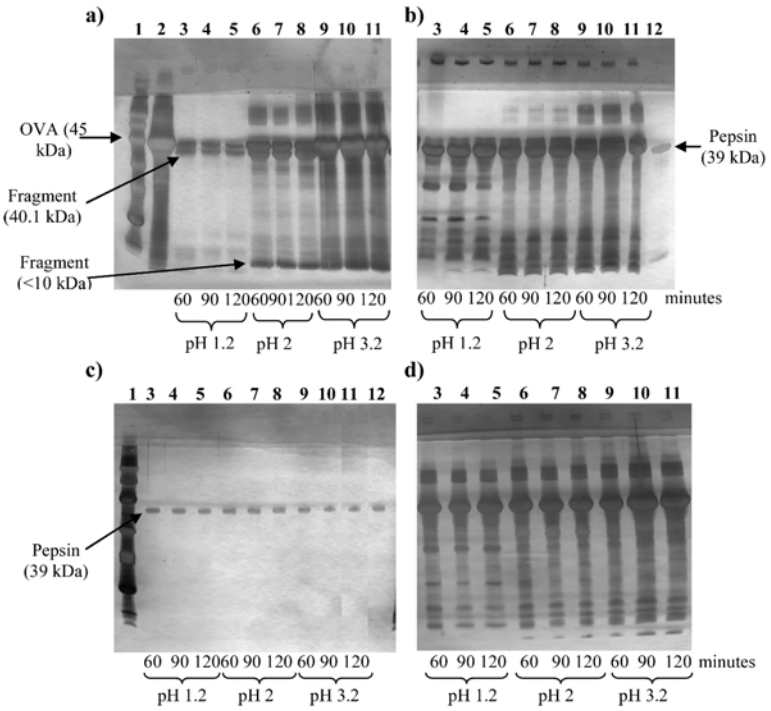

Figure 1. SDS-PAGE analysis of ovalbumin (OVA) digested with pepsin at enzyme/substrate ratios of 1:20 (**a**) and 3:1 (**b**) at different pH values and hydrolysis times and of pepsin at the same concentration as in plates **a** (**c**) and **b** (**d**), but without substrate. Lane 1: molecular mass markers; lane 2: OVA; lanes 3−5: digestions at pH 1.2 during 60, 90, and 120 min; lanes 6−8: digestions at pH 2 during 60, 90, and 120 min; lanes 9−11: digestions at pH 3.2 during 60, 90, and 120 min; lane 12: pepsin.

buffer (TGS), pH 8.3, at 150 V. The gel was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min. The semidry transfer took place in a Trans-Blot SD (Bio-Rad, Richmond, CA, USA) for 30 min at 15 V. Then, the nitrocellulose membrane was blocked with Tris Buffer Saline with Tween 20 (TBST, casein 1% w/v, Tween 20, 0.02% v/v), pH 7.6, for 60 min. The membrane was washed with TBST and dipped into a human serum (25.1 KU/L IgE), 1:20 diluted in TBST-casein (0.1%), overnight at 4 °C. After a new wash, it was incubated overnight at room temperature with HRP-conjugated antihuman IgE antibody (1:500 in TBST). Finally, the membrane was rinsed and detection with chemiluminescence was developed with luminol (Novagen, Darmstadt, Germany). Image acquisition (exposure time 10 min) was performed using the VersaDoc Imaging System (Bio-Rad).

RESULTS AND DISCUSSION

Simulated Gastric Digestion: Influence of the pH, Enzyme to Substrate Ratio, and Presence of Phosphatidylcholine. The SDS-PAGE analysis of OVA incubated with pepsin at an enzyme/substrate ratio of 1:20 and different pHs (**Figure 1a**) showed that pepsin hydrolysis increased with the incubation time, being much faster at the lowest pH. Using the sensitive silver staining, intact OVA was detectable at all pHs even after 120 min of incubation, although it could not be detected with Coomassie Blue after 60 min of hydrolysis at pH 1.2 (data not shown). In agreement with our results, OVA has been reported to be very stable to pepsin at enzyme to protein ratios close to 1:20 (*15*). This stability decreases at higher ratios (*5*), which points at the importance of the assay conditions when trying to correlate pepsin resistance with the allergenic potential of proteins (*16*). Although there is not a general agreement on the physiological enzyme to protein ratio, which might also be subjected to considerable intra- and interindividual variations, it seems clear that, in an in vivo situation, protein would always exceed pepsin (*16*). However, most studies on protein digestibility and, more specifically, OVA digestibility have used high enzyme to substrate ratios, such as 19:1 (*17*), 13:1 (*5*), 8:1 (*18*), or 3:1 (*8*, *19*). In order to check the effect of a higher pepsin concentration, we also tried an enzyme/substrate ratio of 3:1 (**Figure 1b**). In this case, OVA was detected only at pH 3.2 and only traces of intact protein were visible after 60 min of digestion at lower pHs. RP-HPLC analysis confirmed the absence of OVA after 90 min of hydrolysis at pH 1.2 and 2 at an enzyme to substrate ratio of 3:1 (results not shown). This is in argument with a collaborative study between nine laboratories (*8*) using a 3:1 pepsin/protein ratio that revealed that the disappearance of intact OVA and its fragments was not influenced by the pH value (either 1.2 or 2). However, according to our results, the pH seemed to be more relevant when lower relative amounts of enzyme were used. Although pepsin exhibits optimum activity over broad pH range (between 1.2 and 3.5), our results show that, at an enzyme to substrate ratio of 1:20, pH 2 did not particularly favor the enzyme activity. It is assumed that pH 1.5−2 prevails in the fasted stage of the stomach of healthy adults, but this value can increase to above 4 after food intake (*20*). Furthermore, the acidity of the infant stomach is much less, with a pH of around 4, what may lead to a poor and slow degradation of allergenic epitopes (*21*). Therefore, in children or adults with impaired stomach function, a slower and less efficient degradation of OVA could occur at pH higher than 1.2, at pepsin concentrations mimicking physiological conditions that could be of importance in terms of enhancing the sensitization or allergenic capacity of this protein.

It should be noted that the use of the sensitive silver staining made it difficult to interpret the electrophoretic pattern around 45 kDa, because both OVA and pepsin migrate closely in this area. To check this point, as well as to detect any interferences arising from the autodigestion of pepsin, the enzyme was incubated under the same conditions but without substrate. At the

lowest concentration, pepsin appeared as a faint band in the silver-stained gel and no shelf-degradation products were visible (**Figure 1c**). However, at the highest concentration, it became evident that most of the bands generated were fragments derived from pepsin autodigestion (**Figure 1d**). Thus, the main degradation products arising from pepsin action on OVA were more easily detected at the lowest enzyme/substrate ratio. These were a band of around 40 kDa of molecular mass that was quickly generated upon digestion and a band of less than 10 kDa that was observed even after 120 min of digestion at all pHs, which could correspond to an OVA fragment very resistant to pepsin (*9*) (**Figure 1a**). Thomas et al. (*8*); Dearman et al. (*18*), and Takagi et al. (*19*) described a similar digestion pattern, with the formation of two hydrolysis fragments of 40.1 and 4.1 kDa.

The 40.1 kDa band was sliced from the gel and subjected to trypsinolysis, ESI-MS/MS, and MALDI TOF-TOF analysis. **Figure 2** shows the peptide fragments identified in the primary sequence of OVA. According to Kitabatake et al. (*22*) pepsin cleaves OVA between His_{22} and Ala_{23}, this peptide bond being the only one cleft at pH 4. However, Tatsumi and Hirose (*23*) claimed that, even at pH 2, OVA undergoes a limited proteolysis at Ala_{351}, close to the canonical serpin cleavage site Ala_{352}–Ser_{353}. The fact that no peptides corresponding to the N-terminal region of OVA were found by mass spectrometry points out that the 40.1 kDa fragment might correspond to Ala_{23}–Pro_{385}. The formation of this fragment agrees with the results of a study on the effects of pepsinolysis of OVA on the surface hydrophobicity of the degradation products, carried out by Mine et al. (*24*).

1 GSIGAASMEF CFDVFKELKV HHANENIFYC PIAIMSALAM VYLGAKDSTR
51 TQINKVVRFD KLPGFGDSIE AQCGTSVNVH SSLRDILNQI TKPNDVYSFS
101 LASRLYAEER YPILPEYLQC VKELYRGGLE PINFQTAADQ ARELINSWVE
151 SQTNGIIRNV LQPSSVDSQT AMVLVNAIVF KGLWEKAFKD EDTQAMPFRV
201 TEQESKPVQM MYQIGLFRVA SMASEKMKIL ELPFASGTMS MLVLLPDEVS
251 GLEQLESIIN FEKLTEWTSS NVMEERKIKV YLPRMKMEEK YNLTSVLMAM
301 GITDVFSSSA NLSGISSAES LKISQAVHAA HAEINEAGRE VVGSAEAGVD
351 AASVSEEFRA DHPFLFCIKH IATNAVLFFG RCVSP

Figure 2. Primary ovalbumin sequence. Fragments in bold are the peptides recognized by mass spectrometry following in-gel trypsin hydrolysis. Underlined residues correspond to sequences identified by ESI-MS/MS and residues in italics to sequences identified by MALDI-TOF/TOF.

To assess the effect of other factors that may play a role in physiological digestion, such as the interactions between proteins and lipids (*25*), in vitro gastric digestion was also performed in the absence and presence of PC. As shown in **Figure 3a** and **b**, no effect of PC on OVA resistance to digestion was found. PC is secreted by the gastric mucosa and also occurs in the bile. The diet is also a supply of PC, and for instance, there is a high concentration of PC in egg yolk (aprox 1.7 mmol). Moreno et al. (*7*) reported for the first time that the interaction of certain proteins, such as α-La, with physiological surfactants can retard their proteolysis during gastric digestion, although other proteins are unaffected by the presence of PC during pepsin hydrolysis. The protective effect of PC on pepsinolysis was attributed to α-La adopting a partially unfolded state at acidic pH that would favor its partial penetration into PC vesicles. However, the digestion of a protein very resistant to pepsin, such as the 2S albumin from Brazil nut, with a compact 3D structure, is not affected by the presence of PC in SGF at pH 2.5 (*6*). Similarly, addition of PC does not affect the resistance of β-Lg to pepsin at pH 2.5 (*26*, *27*).

Simulated Duodenal Digestion: Influence of Lipases, Bile Salts, and Phosphatidylcholine. The samples obtained after 60 min of an in vitro gastric digestion with pepsin (enzyme/substrate 1:20) at pH 2 were further subjected to a process mimicking duodenal digestion with trypsin and α-chymotrypsin (*6*, *7*). **Figure 4a** shows that both intact OVA and its 40.1 kDa fragment resisted hydrolysis by these enzymes for at least 60 min. According to Fu et al. (*5*), OVA is stable to SIF (simulated intestinal fluid, consisting of pancreatin in alkaline medium). However, Takagi et al. (*19*) reported that the original band of OVA rapidly decreases, while a small amount of the fragment band (40.1) persists for 120 min. As

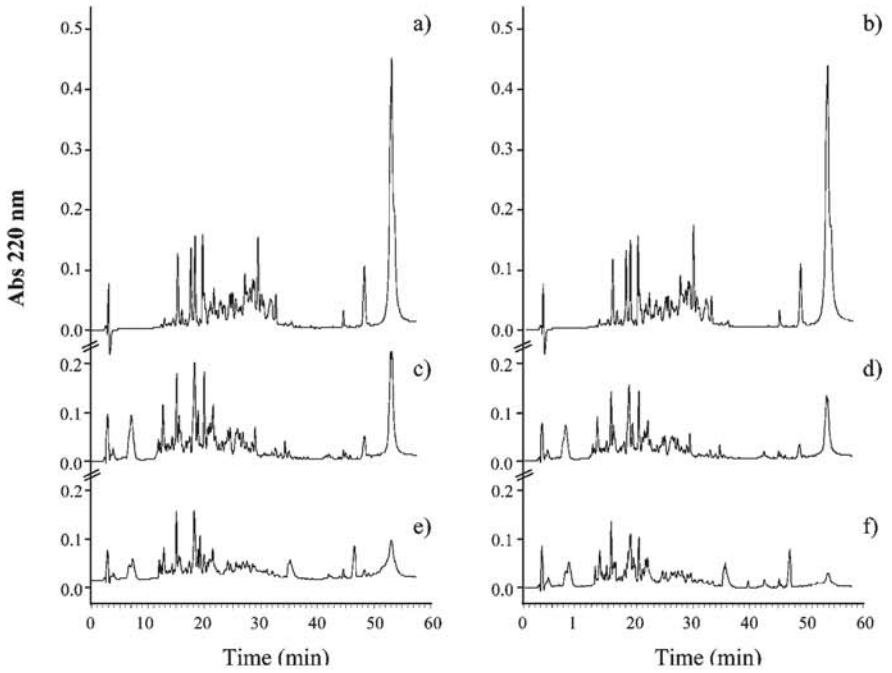

Figure 3. RP-HPLC chromatograms of ovalbumin (OVA) hydrolyzed under different conditions. In vitro gastric digestions with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min, in the absence (**a**) and presence (**b**) of phosphatidylcholine (PC), followed by in vitro duodenal digestions with Corolase PP for 60 min: without BS (BS) and PC (**c**); without BS but with PC (**d**); with BS but without PC (**e**); and with BS and PC (**f**).

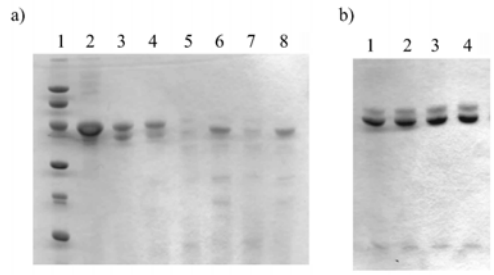

Figure 4. (**a**) SDS-PAGE analysis of ovalbumin (OVA) digests under different conditions. Lane 1: molecular mass markers; lane 2: OVA; lane 3: OVA digested with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min; lane 4: duodenal digestion with trypsin and chymotrypsin for 60 min; lanes 5−8: duodenal digestions with Corolase PP for 60 min with lipases and bile salts (BS) (lane 5), with lipases but without BS (lane 6), without lipases but with BS (lane 7); and without lipases or BS (lane 8). (**b**) SDS-PAGE analysis of OVA digested with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min incubated for 5 (lane 1), 10 (lane 2), and 20 (lane 3) min in duodenal media containing lipases and BS but without proteases.

is the case in SGF, in vitro hydrolysis in SIF can be greatly influenced by the amount and type of enzymes used. In fact, Corolase PP, an enzyme preparation from the pig pancreas gland that in addition to trypsin and chymotrypsin contains amino and carboxypeptidase activities, caused a more extensive proteolytic degradation of both OVA and its fragment (**Figure 4a**, lanes 5−8). In subsequent experiments, Corolase PP and an incubation time of 60 min were used to evaluate the influence of BS, PC, and lipases in the duodenal medium on OVA digestibility.

Parallel in vitro duodenal digestions, following gastric digestion without PC, were carried out, giving rise to four digestion samples: in the presence of all of the duodenal components, in the absence of BS, in the absence of lipases, and in the absence of BS and lipases. Lipases did not prove to have any effect on protein digestibility; however, SDS-PAGE (**Figure 4a**) and RP-HPLC analyses (**Figure 3e** and **f**) revealed that BS considerably enhanced the hydrolysis of the intact protein and the large fragment generated after pepsin digestion (which coelutes with OVA in this system). Gass et al. (*28*) recently reported that BS accelerate the cleavage by trypsin and chymotrypsin of some but not all of a spectrum of dietary proteins. According to these authors, 10 mM of a bile acid mixture (containing taurocholate and glycodeoxycholate) considerably promotes the digestion rates of β-Lg, myoglobulin, and BSA, probably through the destabilization of their tertiary structure. However, unlike the present results, Gass et al. (*28*) reported that chicken OVA remains unaffected by the BS.

To elucidate whether BS could have any effect on protein solubility, we incubated a gastric digest with the duodenal digestion mixture without Corolase PP. As shown in **Figure 4b**, exposure to the simulated duodenal conditions without proteases did not alter the band pattern. On the other hand, it has been proven that bile acids do not have any effect on the activity profile of trypsin or chymotrypsin (*28*).

According to Gass et al. (*28*), physiologic bile acids can exert their effect as individual molecules (below the critical micelle concentration, 3.5 mM), but this is more pronounced when they act as micelles. The concentration of amphiphilic bile components, including BS and PC, increases after a meal. In that sense, the composition of our duodenal digestion mixture resembles that described for a fed state SIF, which can be simulated with 15 mM sodium taurocholate and 3.75 mM PC, while a simulated fasted state SIF contains 3 mM sodium taurocholate and 0.75 mM PC (*29*).

Gastric digests obtained in the presence of PC were also subjected to duodenal digestion. RP-HPLC analysis revealed a somehow higher proteolysis degree when PC was present, particularly in combination with the bile salt mixture (**Figure 3d** and **f**). The protective effect of PC on gastric digestion of α-La reported by Moreno et al. (*7*) is lost in a subsequent duodenal digestion, an observation that the authors attributed to the disruption of the vesicular structure of PC by the BS present in the duodenal digestion mix. On the other hand, PC was found to protect β-Lg from degradation under duodenal conditions (*26*). In this latter case, the protective effect could be ascribed not to the insertion of the protein into the PC vesicles, as the bile salt-PC components are exclusively present as mixed micelles in this system, but to the lipids binding to the secondary fatty acid binding site of β-Lg, which would block the proteinase action for steric reasons (*26*).

Interaction of Ovalbumin with Phosphatidylcholine and Bile Salts. The conformational state of OVA was analyzed by circular dichroism (CD) spectroscopy at pH 2 (in the absence and presence of PC) and pH 7 (in the absence and presence of PC and/or BS). As for the far-UV spectra (**Figure 5a**), the secondary structure analysis revealed a slight difference between both pH conditions, showing an approximately 5% α helix loss and a 5% β strand gain when changing from an acidic to a neutral environment. No effect on the secondary structure motif composition was observed by the addition of PC and/or BS at any pH tested. The featureless near-UV spectrum around zero for all wavelengths obtained at pH 2 (**Figure 5b**) clearly indicated the tertiary structure loss, typical of the molten globule state adopted by OVA at this pH. At pH 7, the characteristic spectrum of the protein was observed. These data reflected very different tertiary structures at both pH values, but no influence of PC, BS, or their combination.

The CD spectra of OVA show that its secondary structure content is essentially the same at pH 2 and pH 7, but the native tertiary interactions are almost completely disrupted at acidic pH (*23*, *30*). α-La, which is claimed to be protected from pepsin hydrolysis by insertion into PC vesicles, in the absence of PC undergoes a significant proteolysis by pepsin in the molten globule state induced by acidic pH (*7*). However, the molten globule-like state of OVA induced at pH 2 is resistant to pepsin digestion under conditions similar to those used for α-La, suggesting a much more limited disorder in the tertiary structure (*23*). The observation that OVA assumes a highly ordered molten globule conformation at pH 2.2, with the intrachain disulfide bond adding a high conformational stability to its structure (*30*), may explain why it does not adopt enough flexibility to penetrate into PC vesicles.

Fluorescence experiments were performed to evaluate any conformational change that could increase the exposure of Tyr and Trp residues to the solvent as a consequence of the interaction of OVA with PC or BS. The fluorescence intensity of OVA at pH 2 was lower than at pH 7, but the maximum wavelength of 334 nm was unchanged (**Figure 6a**). The lower intensity at acidic pH is due to the quenching effect exerted by the protonated Glu residues located in the vicinity of the three Trp residues in the OVA sequence (*31*). Addition of PC decreased the fluorescence intensity at pH 2 and increased the fluorescence intensity at pH 7, but did not change the emission maximum. The titration experiments (**Figure 6b**) showed that the fluorescence intensity at the emission maximum progressively changed with the concentration of added PC, particularly at pH 2. Fluorescence quenching upon PC addition at pH 2 could be due to binding of PC to the protein, which would cause the transfer of resonant energy between the excited aromatic rings and the ligand, while fluorescence increase at pH 7

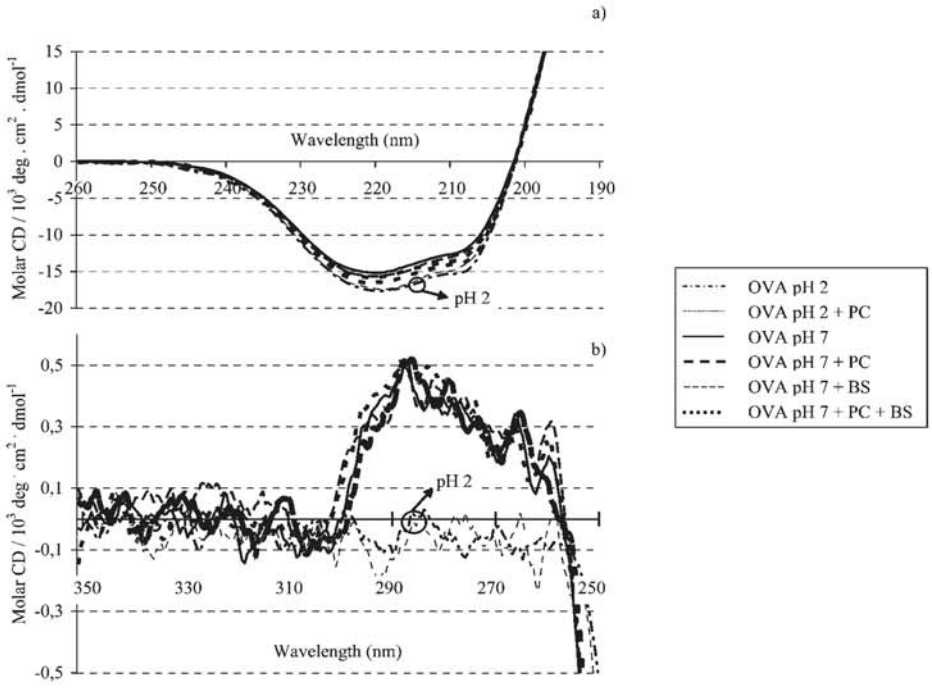

Figure 5. Circular dichroism spectra in the far (**a**) and the near (**b**) UV region of ovalbumin (OVA) at pH 2 in the absence and presence of phosphatidylcholine (PC) and at pH 7 in the absence and presence of PC, bile salts (BS), and their combination.

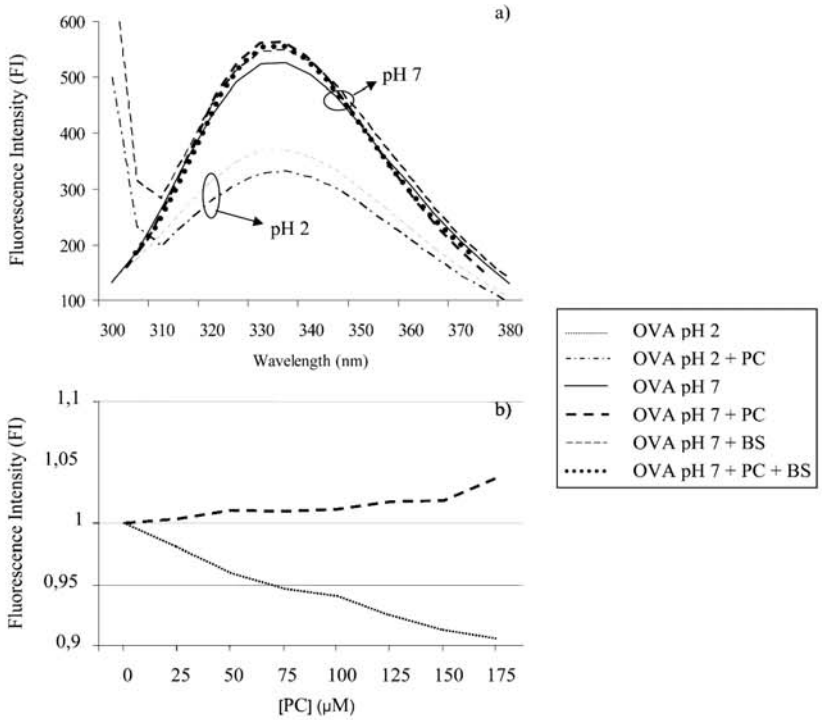

Figure 6. (**a**) Fluorescence spectra of ovalbumin (OVA) at pH 2 in the absence and presence of phosphatidylcholine (PC) and at pH 7 in the absence and presence of PC, bile salts (BS), and their combination. (**b**) Corrected OVA titration curves with PC at pH 2 and pH 7.

could reflect a decrease in polarity in the neighborhood of the excited residues caused by water displacement by the ligand (*32*). Mine et al. (*33*) reported that OVA can interact with PC vesicles at PC/OVA ratios from 10 to 40 M:M, showing a higher affinity for PC at acidic than at basic pH.

As has been described for other proteins, a simple electrostatic attraction of OVA with zwitterionic phospholipids, such as PC, would provide a loose association, so that binding would be reinforced at a low pH, when the protein would normally unfold (*34*−*36*). In fact, the interaction between OVA and PC

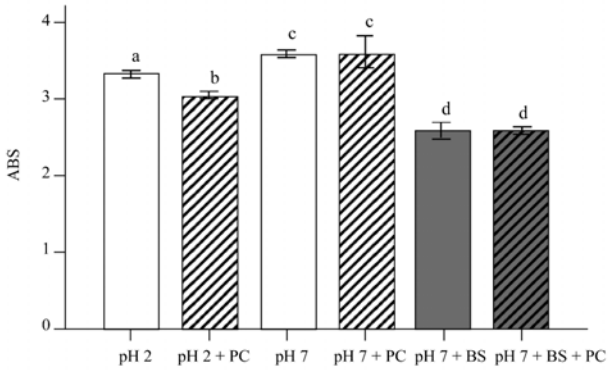

Figure 7. ELISA response against IgG of ovalbumin (OVA) at pH 2 in the absence and presence of phosphatidylcholine (PC) and at pH 7 in the absence and presence of PC, bile salts (BS) and their combination. Error bars correspond to 95% confidence intervals. Different letters above the bars indicate significant differences ($P < 0.05$).

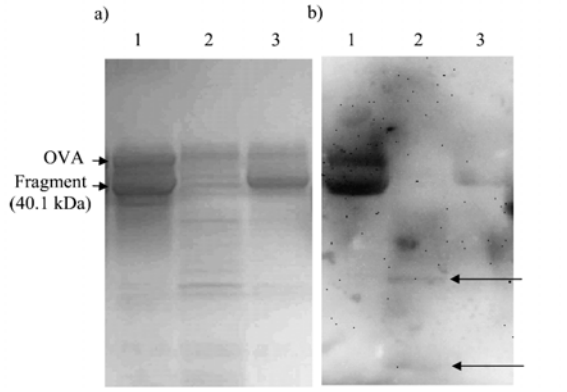

Figure 8. SDS-PAGE (**a**) and Western blot (**b**) of ovalbumin (OVA) digests. Lane 1: OVA digested with pepsin at an enzyme/substrate ratio of 1:20, at pH 2, for 60 min; lane 2: subsequent duodenal digestion with Corolase PP for 60 min in the presence of bile salts; lane 3: duodenal digestion with Corolase PP for 60 min in the absence of bile salts.

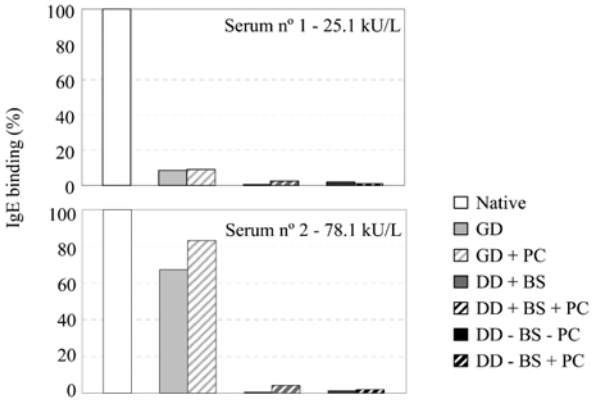

Figure 9. IgE binding estimated by inhibition ELISA, with two human sera of patients allergic to egg, of native ovalbumin (OVA) and its gastric digests at a pepsin/substrate ratio of 1:20, at pH 2, for 60 min (GD) in the presence (+ PC) and absence of phosphatidylcholine (−PC) and of duodenal digests with Corolase PP for 60 min (DD), in the presence (+BS) and absence of bile salts (−BS) and their combinations with PC.

was promoted at acidic pH, when the surface hydrophobicity of the protein was higher. However, the observation that PC did not change its susceptibility to proteolysis by pepsin suggests that, by virtue of its high conformational stability at acidic pH, either OVA did not became inserted into PC vesicles, or such insertion did not provide the protein with extra protection on incubation with the enzyme.

At neutral pH and in the absence of BS, electrostatic binding of OVA to PC may cause OVA to associate with the vesicles surface, increasing the exposure of the protein to pancreatic proteinases. Mogensen et al. (*36*) found that Bet v1, the major allergen from birch tree pollen, interacts with micelles forming lysophospholipids in a pH-depending manner. At pH 3.9, Bet v1 is inserted deeply into the membrane, and this prevents a general degradation of the protein on incubation with pepsin. At pH 7.2, Bet v1 associates to the membrane without loosing its integrity, and this makes it more sensitive to proteolytic degradation with trypsin.

BS (sodium taurocholate and sodium glycodeoxycholate were evaluated separately) did not exert any noticeable effect on the fluorescence emission of OVA (data not shown). In any case, our fluorescence experiments could not rule out the binding of protein to bile acids, as the maximum concentration used was below 0.5 mM. However, IgG binding to the protein, basically unchanged in the presence of PC at pH 7, was significantly ($P < 0.05$) decreased in the presence of BS (**Figure 7**), which points to structural changes due to mutual interaction. It is suggested that OVA could associate with the surfactants or with the mixed bile salt-PC micelles present, an effect that has been proposed to occur to proteins in the duodenum (*25*), increasing its exposure to pancreatic proteinases.

Immunoreactivity of OVA and Its Digests. Immunoblotting of gastric and duodenal digests of OVA in the absence and presence of BS is shown in **Figure 8**. Intact OVA and its 40.1 kDa fragment exhibited a considerable IgE-binding activity, which virtually disappeared after duodenal digestion and, particularly, in the presence of BS because of the higher digestibility promoted under those conditions. The immunoblotting suggests that the duodenal digestion products could still retain IgE-binding epitopes (indicated by arrows in **Figure 8b**), and this was further checked by competition ELISA with sera from patients allergic to eggs (**Figure 9**).

IgE binding decreased, depending on the patient serum, after in vitro gastric digestion and was further reduced after duodenal digestion. In vitro duodenal digestion under all conditions resulted in low reactivity against IgE from sera of allergic patients, although the detectable IgE binding response suggested the presence of peptides containing intact binding sites recognizable by serum IgE. It remains to be elucidated whether these fragments contain single IgE epitopes or more than one IgE binding site, which would make them capable of cross-linking IgE bond receptors and cause cell degranulation. Furthermore, the effect of surfactants on the proteolytic stability of the degradation products deserves further investigation. Thus, it has been suggested that during the digestion of whole egg, hydrolysis fragments of OVA could emulsify with the egg yolk PC, which would protect them from digestion and increase their intestinal absorption (*37*).

CONCLUSIONS

OVA was very resistant to pepsin action at an enzyme/substrate ratio that would resemble a physiological situation (1:20 w/w, 172 units/mg), at pH values equal to or above 2, typical of a fed state and, particularly, of the stomach of children. Under these conditions, the intact protein and its main degradation product, a 40 kDa fragment resulting from cleavage at the

N-terminal side, persisted for at least 2 h of digestion. The presence of the physiological surfactant PC did not change the susceptibility of OVA to proteolysis with pepsin. Fluorescence experiments showed that OVA interacted with PC vesicles, particularly at acidic pH, when the protein adopts a molten globule state devoid of tertiary structure interactions, but when it likely maintains a high degree of conformational stability, resisting pepsin action.

The presence of BS at physiological concentrations, typical of the duodenal fed state, considerably increased the proteolysis of OVA by a mixture of pancreatic enzymes. In the presence of BS, the CD spectra of OVA did not show any sign of tertiary structure destabilization that could make the protein more prone to proteinase action; however, antibody binding to the protein was considerably decreased in the presence of BS, what points to structural changes due to mutual interaction. The addition of PC made OVA even more sensitive to proteolytic degradation under duodenal conditions, suggesting that OVA could associate with the surfactants or with the mixed bile salt-PC micelles present in the duodenum, increasing the exposure of the protein to pancreatic proteinases.

Immunoreactivity against IgE from sera of allergic patients was retained after in vitro gastric digestion, depending on the reactivity of the sera, but it decreased considerably after in vitro duodenal digestion under all conditions. Nevertheless, the persistence of peptides containing intact binding sites recognizable by serum IgE could not be discarded.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Mine, Y.; Yang, M. Concepts of hypoallergenicity. In *Bioactive Egg Compounds*; Huopalahti, R., López-Fandiño, R., Anton, M., Schade, R., Eds.; Springer-Verlag: Berlin Heidelberg, Germany, 2007; pp 145−158.

(2) Moreno, J. Gastrointestinal digestion of food allergens: effect on their allergenicity. *Biomed. Pharmacother.* **2007**, *61*, 50–60.

(3) Vieths, S.; Reindl, J.; Muller, U.; Hoffmann, A.; Haustein, D. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *Eur. Food Res. Technol.* **1999**, *209*, 379–388.

(4) Kenna, J. G.; Evans, R. M. Digestibility of proteins in simulated gastric fluid. *Toxicologist* **2000**, *54*, 141.

(5) Fu, T. -J.; Abbott, U. R.; Hatzons, C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid-A comparative study. *J. Agric. Food Chem.* **2002**, *50*, 7154–7160.

(6) Moreno, F. J.; Mellon, F. A.; Wickham, M. S. J.; Bottrill, A. R.; Mills, E. N. C. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. *FEBS J.* **2005a**, *272*, 341–352.

(7) Moreno, F. J.; Mackie, A. R.; Mills, E. C. N. Phospholipid interactions protect the milk allergen α-lactalbumin from proteolysis during in vitro digestion. *J. Agric. Food Chem.* **2005b**, *53*, 9810–9816.

(8) Thomas, K.; Aalbers, M.; Bannon, G. A.; Bartels, M.; Dearman, R. J.; Esdaile, D. J.; Fu, T. J.; Glatt, C. M.; Hadfield, N.; Hatzos, C.; Hefle, S. L.; Heylings, J. R.; Goodman, R. E.; Henry, B.; Herouet, C.; Holsapple, M.; Ladics, G. S.; Landry, T. D.; MacIntosh, S. C.; Rice, E. A.; Privalle, L. S.; Steiner, H. Y.; Teshima, R.; van Ree, R.; Woolhiser, M.; Zawodny, J. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regul. Toxicol. Pharmacol.* **2004**, *39*, 87–98.

(9) Quirós, A.; Chicón, R.; Recio, I.; López-Fandiño, R. The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chem.* **2007**, *104*, 1734–1739.

(10) Canals, F.; Colomé, N.; Ferrer, C.; Plaza-Calonge, M. C.; Rodríguez-Manzaneque, J. C. Identification of substrates of the extracellular protease ADAMTS1 by DIGE proteomic analysis. *Proteomics* **2006**, *6*, S28–S35.

(11) Barbana, C.; Pérez, M. D.; Sánchez, L.; Dalgalarrondo, M.; Chobert, J. M.; Haertlé, T.; Calvo, M. Interaction of bovine α-lactalbumin with fatty acids as determined by partition equilibrium and fluorescence spectroscopy. *Int. Dairy J.* **2006**, *16*, 18–25.

(12) Whitmore, L.; Wallace, B. A. DICHROWEB: an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res.* **2004**, *32*, W668–673.

(13) Manavalan, P.; Johnson, W. C.Jr. Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. *Anal. Biochem.* **1987**, *167*, 76–85.

(14) Lees, J. G.; Miles, A. J.; Wien, F.; Wallace, B. A. A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics* **2006**, *22*, 1955–1962.

(15) López-Expósito, I.; Recio, I.; Belloque, J.; Chicon, R.; Alonso, E.; López-Fandiño, R. Changes induced by high pressure on ovalbumin proteolysis and effects on the allergenicity of the hydrolysates. *J. Agric. Food Chem.* **2008**, *56*, 11809–11816.

(16) Taylor, S. L. Comment on digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid-A comparative study. *J. Agric. Food Chem.* **2003**, *51*, 5183–5184.

(17) Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **1996**, *14*, 1269–1273.

(18) Dearman, R. J.; Caddick, H.; Stone, S.; Kenna, J. G.; Basketter, D. A.; Kimber, I. Immunogenic properties of rapidly digested food proteins following gavage exposure to mice: a comparison of ovalbumin with a potato acid phosphatase preparation. *Food Chem. Toxicol.* **2002**, *40*, 625–633.

(19) Takagi, K.; Teshima, R.; Okunuki, H.; Sawada, J.-I. Comparative study of in vitro digestibility of food proteins and effect of preheating on the digetion. *J. Biol. Pharm. Bull.* **2003**, *l26*, 969–973.

(20) Kopper, R. A.; Odum, N. J.; Sen, M.; Helm, R. M.; Stanley, J. S.; Burks, A. W. Peanut protein allergens: the effect of roasting on solubility and allergenicity. *Int. Arch. Allergy Immunol.* **2005**, *136*, 16–22.

(21) Schmidt, D. G.; Meijer, R. J. G. M.; Slangen, C. J.; Van Beresteijn, E. C. H. Raising the pH of the pepsin-catalyzed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolysates. *Clin. Exp. Allergy* **1995**, *25*, 1007–1017.

(22) Kitabatake, N.; Indo, K.; Doi, E. Limited proteolysis of ovalbumin by pepsin. *J. Agric. Food Chem.* **1988**, *36*, 417–420.

(23) Tatsumi, E.; Hirose, M. Highly ordered molten globule-like state of ovalbumin at acidic pH: Native-like fragmentation by protease and selective modification of Cys^{367} with dithipyridine. *J. Biochem.* **1997**, *122*, 300–308.

(24) Mine, Y.; Kazuhiro, C.; Masahiro, T. Effects of a limited proteolysis of ovalbumin on interfacial adsorptivity studied by 31p nuclear magnetic resonance. *J. Agric. Food. Chem.* **1992**, *40*, 22–26.

(25) Burnett, G. R.; Wickham, M.; Fillery-Travis, A.; Robertson, J. A.; Belton, P. S.; Gilbert, S. M.; Tatham, A. S.; Shewry, P. R.; Mills, E. N.C. Interaction between protein allergens and model gastric emulsions. *Biochem. Soc. Trans.* **2002**, *30*, 916–918.

(26) Mandalari, G.; Mackie, Alan, M.; Rigby, N. M.; Wickham, M. S. J.; Mills, E. N. C. Physiological phosphatidylcholine protects bovine β-lactoglobulin from simulated gastrointestinal proteolysis. *Mol. Nutr. Food Res.* **2009**, *53*, S131–S139.

(27) Macierzanka, A.; Sancho, A. I.; Mills, E. N. C.; Rigby, N. M.; Mackie, A. R. Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin. *Soft Matter* **2009**, *5*, 538–550.

(28) Gass, J.; Vora, H.; Hofman, A. G.; Gray, G. M.; Khosla, C. Enhancement of dietary protein digestion by conjugated bile acids. *Gastroenterology* **2007**, *133*, 16–23.

(29) Kostewicz, E. S.; Brauns, U.; Becker, R.; Dressman, J. B. Forecasting the oral absorption behavior of poorly soluble weak bases using solubility and dissolution studies in biorelevant media. *Pharm. Res.* **2002**, *19*, 345–349.

(30) Tatsumi, E.; Yoshimatsu, D.; Hirose, M. Conformational state of disulfide-reduced ovalbumin at acidic pH. *Biosci., Biotechnol., Biochem.* **1999**, *7*, 1285–1290.

(31) Zemser, M.; Friedman, M.; Katzhendler, J.; Greene, L. J.; Minsky, A.; Gorinstein, S. Relationship between functional properties and structure of ovalbumin. *J. Prot. Chem.* **1994**, *13*, 261–274.

(32) Muresan, S.; van der Vent, A.; de Wolf, F. A. Interaction of β-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: nonlinear quenching effects related to protein-protein association. *J. Agric. Food Chem.* **2001**, *49*, 2609–2618.

(33) Mine, Y.; Kobayashi, H.; Chiba, K.; Tada, M. ^{31}P NMR study on the interfacial adsorptivity of ovalbumin promoted by lysophosphatidylcholine and free fatty acids. *J. Agric. Food. Chem.* **1992**, *40*, 1111–1115.

(34) Hanssens, I.; Ceunebroeck, J.; Pottel, H.; Preaux, G.; van Cauwelaert, F. Influence of the protein conformation on the interaction between vesicle of α-lactalbumin and dimyristoylphosphatidylcholine. *Biochim. Biophys. Acta* **1985**, *817*, 154–164.

(35) Cawthern, K. M.; Permyakov, E.; Berliner, L. J. Membrane-bound states of alpha-lactalbumin: implications for the protein stability and conformation. *Protein Sci.* **1996**, *5*, 1394–405.

(36) Mogensen, J. E.; Ferreras, M.; Wimmer, R.; Petersen, S. V.; Enghild, J. J.; Otzen, D. E. The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. *Biochemistry* **2007**, *46*, 3356–3365.

(37) Fujita, H.; Sasaki, R.; Yoshikawa, M. Potentiation of the antihypertensive activity of orally administered ovokinin, a vasorelaxing peptide derived from ovalbumin, by emulsification in egg phosphatidyl-choline. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 2344–2345.

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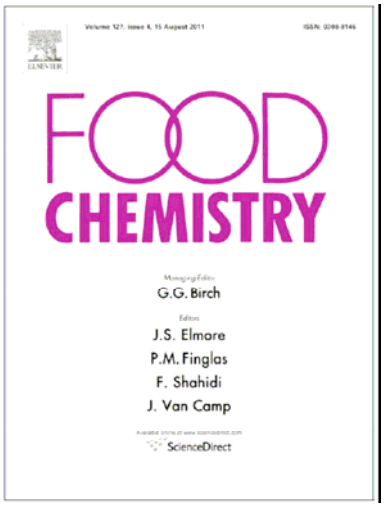

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ABSTRACT

This paper examines lysozyme (LYS) behaviour upon *in-vitro* digestion, mimicking different conditions in the stomach and intestine, and assessing the effect of natural surfactants, such as phosphatidylcholine (PC) or bile salts (BS), on hydrolysis and residual immunogenicity of the digests. The hydrolysis pattern of LYS was compared to that of α-lactalbumin (LA). Hydrolysis of LYS only occurred at low pH. PC slightly increased its resistance to pepsinolysis. A similar behaviour was found for LA. Circular dichroism revealed that the more rigid structure of LYS, as compared with that of LA, could protect it from proteolysis at acidic pH and fluorescence spectra suggested that, at acidic pH, both proteins associated to PC films. The gastric digests of LYS showed high IgE-binding capacity using sera from egg-allergic patients. On the other hand, it was found that LYS precipitated under conditions that simulated a duodenal environment, mainly due to the presence of BS.

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1. Introduction

Lysozyme (LYS) is, together with lactoferrin, one of the most extensively studied antibacterial milk proteins (López-Exposito & Recio, 2006). LYS is present in milk from different species, but also in many biological fluids and tissues, such as human milk, tears and saliva. Its bactericidal effect partially depends on its lytic activity on the cell wall of Gram-positive microorganisms. Many attempts have been made to broaden its antimicrobial activity to include Gram-negative bacteria, such as the use of thermal treatments to partially denature the protein, the linkage of hydrophobic ligands to increase its hydrophobicity or the conjugation to polysaccharides through controlled Maillard reaction (Pellegrini et al., 1997). In addition to conformational changes, proteolysis of LYS has been shown to produce peptides able to induce a non-enzymatic bacterial inactivation and are, therefore, active against Gram-positive and Gram-negative bacteria (Ibrahim, Inazaki, Abdou, Aoki, & Kim, 2005; Mine, Ma, & Lauriau, 2004). Other biological functions of LYS, such as immunomodulatory, antiviral and anti-inflammatory, have been reported (Lesnierowski & Kijowski, 2007).

It can be presumed that milk LYS can act physiologically as a bactericidal agent, although the existence of a definite protective role is not yet known. In fact, the high level of LYS in human milk (which is 100 times higher than that of bovine milk, which contains from 0.13 to 0.32 mg/L of LYS) could be of relevance (Fox & Kelly, 2006). In any case, exogenous LYS is widely added to several cheese varieties to prevent the growth of *Clostridium*, which causes off-flavours and late blowing and its use, in combination with minimal processing techniques, has been proposed to extend the shelf life of milk and dairy products (Sobrino-López & Martín-Belloso, 2008).

Hen egg white (probably the richest source of LYS, containing 1–3 g/L) is the main commercial source of LYS used to maintain the quality of food and pharmaceutical products. Members of the LYS superfamily are related proteins, with similar three-dimensional structures but different amino acid sequences, comprising from 123 to 129 amino acids. Egg-white LYS (Gal d 4, with mass of 14.3 kDa, 129 amino acid residues and four disulphide bridges) has been extensively studied as a model protein for structure and biological properties of LYS from other sources. However, LYS is also a major allergen of egg white, although its allergenic potential has not been studied in depth and no relevant epitopes have been identified as yet. Clinical reactions to egg LYS have been described and anti-LYS IgE antibodies are frequently found in egg allergic patients as markers of sensitisation (Mine & Yang, 2008). In fact, the frequent presence of LYS as an additive in dairy products poses a risk to allergic subjects (Iaconelli et al., 2008).

It is generally accepted that resistance to digestion is a common feature to food allergens, although it has also been shown that proteolytic fragments produced during digestion may bind IgE and induce allergic responses in sensitised individuals. Besides, in the case of LYS, digestion can be a physiological process to release peptides that contribute to its *in vivo* defence role. However, available information related to the basis of the resistance of this

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protein to digestion is limited and, sometimes, contradictory. This can be attributed to the fact that *in-vitro* digestion models use inappropriate amounts of proteases or one-step digestion models, disregarding the complex gastric and duodenal composition and the interactions of proteins with other components, such as lipids (Moreno, 2007).

This paper examines LYS behaviour upon *in-vitro* digestion, mimicking different conditions in the stomach and upper intestine, and assessing the effect of natural surfactants, such as phosphatidylcholine (PC) or bile salts (BS), on hydrolysis and residual immunogenicity of the digests. The structural characteristics of LYS that could play a role in its susceptibility to proteolysis under different conditions were studied and compared to those of its homologous protein bovine milk α-lactalbumin (LA), which is more susceptible to proteolysis. Because of its broader availability, hen-egg LYS, which shows 60% homology with human milk LYS, was used. The results obtained regarding egg-white LYS behaviour towards digestion can be relevant in understanding its allergenic potential, of importance when added to dairy products, and the contribution of physiological processes to its *in-vivo* defence role that could be common to other members of the LYS family.

2. Materials and methods

2.1. Gastric and Duodenal digestions

LYS (L2879, chloride form from chicken egg white Grade VI, ~60000 units/mg protein, EC 3.2.1.17, Sigma–Aldrich, St. Louis, MO) was subjected to *in-vitro* gastric digestions at 5 mg/mL final concentration. The digestions were performed in simulated gastric fluid (SGF, 0.35 M NaCl) at different pHs: 1.2, 2.0, 3.2, 4.0 and 4.5, at 37 °C for 120 min, with porcine pepsin (EC 3.4.23.1, 3440 U/mg protein, Sigma–Aldrich) at an enzyme: substrate ratio (E:S) of 1:20, wt/wt (172 U/mg), considered as a physiological ratio (Moreno, Mellon, Wickman, Bottrill, & Mills, 2005a).

Gastric digestions in the presence of the natural surfactant phosphatidylcholine (PC, Sigma–Aldrich), were also assessed. Phospholipid vesicles were prepared by dissolving PC in SGF pH 2.0 (9.58 mg/mL), following Martos, Contreras, Molina, and López-Fandiño (2010).

At least three replicates of each digestion assay were performed and compared by HPLC and SDS–PAGE to ensure the repeatability of the results. Aliquots were taken at different time points, up to 120 min, for analysis. The digestions were stopped by mixing with the SDS-sample buffer for SDS–PAGE analyses, whereas for RP-HPLC and inhibition ELISA, the pH was raised to 7.0 with ammonium bicarbonate to irreversibly inactivate pepsin and, after 10 min of equilibration, the digestion mixtures were centrifuged at 10000*g* and 20 °C for 10 min. The protein concentration of the supernatants was determined by the Kjeldahl method.

Bovine α-lactalbumin (LA, Sigma–Aldrich) was also submitted to *in-vitro* gastric digestions without and with PC under the same conditions. In all cases, at least triplicate digestions were conducted.

Duodenal digestions were performed as previously described (Martos et al. 2010; Moreno, Mackie, & Mills 2005b; Moreno et al., 2005a) on the supernatants of the 60-min gastric digests re-adjusted to pH 6.5, with the addition of: a 0.125 M bile salt (BS) mixture containing equimolar quantities of sodium glycodeoxycholate and sodium taurocholate (Sigma–Aldrich) (6.15 mM final concentration of each salt); 1 M $CaCl_2$ (7.6 mM final concentration); pancreatic porcine lipase (Type VI-S, 111000 U/mg protein, Sigma–Aldrich), at an E:S of 1:3895, wt/wt (28.5 U/mg); pancreatic porcine colipase (Sigma–Aldrich), at an E:S of 1:895, wt/wt; pancreatic bovine trypsin (type I, 10100 U/mg protein, Sigma–Aldrich), at an E:S of 1:238, wt/wt (42.5 U/mg); and pancreatic bovine α-chymotrypsin (type I–S, 58.3 U/mg protein, Sigma–Aldrich), at an E:S of 1:115 wt/wt (0.52 U/mg) in 20.3 mM Bis–Tris. The reactions were carried out at 37 °C for either 30 or 60 min and stopped by heating at 80 °C for 5 min.

2.2. Solubility experiments

The solubility of LYS was examined in SGF at different pHs (1.2, 2, 3.2, 3.6, 3.9, 4.7, 5.1, 6.2, 6.4, 6.8, 7.2 and 7.7) as well as in 10 mM phosphate buffer. Solubility of the intact protein was also assessed in 7.6 mM $CaCl_2$ and 20.3 mM Bis–Tris under simulated duodenal conditions typical of a fasted state (3 mM of each BS and 2.4 mM PC, pH 7.0), of a fed state (9 mM of each BS and 7.2 mM PC, pH 6.0) and of an intermediate state (6 mM of each BS and 4.9 mM PC, pH 6.5) (Kostewicz, Brauns, Becker, and Dressman, 2002; Kaukonen, Boyd, Porter, & Charman, 2004). This last condition was similar to the medium used for duodenal digestion. Solubility was determined by SDS–PAGE and/or RP-HPLC analysis following centrifugation (10000*g*, 20 °C, 10 min).

2.3. SDS–PAGE

SDS–PAGE of the gastric digests was performed using Tris-Tricine ready gels with 16.5% acrylamide (Bio-Rad Laboratories, Hercules, CA). Samples were diluted in Tris-Tricine sample buffer (Bio-Rad). Electrophoresis was carried out at 100 V, for 3 h, at room temperature (RT), in Tris-Tricine SDS running buffer (Bio-Rad). Gels were fixed in a 40% methanol and 10% (wt/vol) acetic acid solution, followed by staining with Coomassie Blue G-250 (Bio-Rad). The kaleidoscope pre-stained standards (Bio-Rad) containing myosin (198 kDa), β-galactosidase (125 kDa), BSA (88 kDa), carbonic anhydrase (37 kDa), STI (31 kDa), LYS (17 kDa) and aprotinin (7 kDa) were used.

SDS–PAGE of the duodenal digests was performed using PhastSystem equipment (Pharmacia, Uppsala, Sweden) using PhastGel Homogeneous 20 polyacrylamide gels and PhastGel SDS-Buffer Strips (Pharmacia) and following the manufacturer's separation and Coomassie staining conditions. The low molecular weight calibration kit for SDS electrophoresis (GE Healthcare, Uppsala, Sweden) containing phosphorylase B (97 kDa), BSA (66 kDa), OVA (45 kDa), carbonic anhydrase (37 kDa), trypsin inhibitor (20.1 kDa), and LA (14.4 kDa), was used.

2.4. RP-HPLC

LYS hydrolysates, at a concentration of 2.4 mg/mL, were analysed using a Hi-Pore® RP-318 (250 × 4.6 mm i.d.) column (Waters, Milford, MA) in a Waters 600 HPLC system. Solvent A was 0.37% (vol/vol) trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) in double-distilled water and solvent B was 0.27% (vol/vol) trifluoroacetic acid in HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions were as in Martos et al. (2010). Detection was at 220 nm and data were processed by using Empower 2 Software (Waters).

2.5. Circular dichroism spectroscopy

CD spectra were recorded in the far (195–260 nm) and near (250–350 nm) UV regions, using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) as described in Martos et al. (2010). LYS and LA were dissolved at 0.2 mg/mL for the analysis in the far-UV region and at 2 mg/mL for the near-UV region, either in SGF (pH 2.0) or in 10 mM phosphate buffer (pH 7.0). PC was added at 0.184 mg/mL or 1.84 mg/mL, respectively, to maintain the protein/phospholipid ratio used during digestion. Buffer blanks were subtracted from each CD spectrum.

2.6. Fluorescence spectroscopy

The interaction between either LYS or LA and PC was studied by fluorescence spectroscopy based on Barbana et al. (2006). Fluorescence spectra between 300 and 380 nm (excitation: 280 nm) were recorded at room temperature on a Shimadzu RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The binding of PC was measured by following the increase in protein fluorescence. The procedure used for titration of LYS and LA was as described in Martos et al. (2010).

2.7. IgG- and IgE-binding by inhibition ELISA

The antigenicity of LYS and its gastric hydrolysates was evaluated by inhibition ELISA using commercial IgG antibodies. Single wells of polystyrene microtitre plates (Corning, Cambridge, MA) were coated with 10 μg/mL of LYS solution in 0.01 M PBS, pH 7.4, and incubated overnight at 6 °C. Afterwards, the plates were washed three times with PBS-Tween 20 (PBST) 0.05% using a Microplate Washer (Nunc, Roskilde, Denmark) and PBST 2.5% was used as saturating agent, to avoid non-specific binding. Then, the plate was blocked for 4 h at RT and washed once.

Serial dilutions of each sample (not less than 10) were incubated (1:1, vol/vol) at RT for 2 h, with horseradish peroxidase (HRP) conjugated-polyclonal anti-LYS raised in rabbit (Abcam, Cambridge, UK), previously diluted 1:10000 in PBST 0.05%, and 50 μL were added to each well. After 2 h of incubation at RT, the plate was washed three times and 50 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (ready-to-use solution; Sigma–Aldrich) were added to each well. Finally, the reaction was stopped with 0.5 M sulphuric acid and the absorbance was measured at 450 nm. A negative control without antibody (native protein in PBST 0.05%) and positive controls (antibody diluted in PBS) were included in each plate.

For human IgE-binding, three individual serum samples from children with clinically allergic symptoms to egg white proteins were used. The sera were collected from the Maternal and Child Gregorio Marañon Hospital (Madrid, Spain). The patients had specific seric IgE levels towards egg white higher than 100 kU/L, as determined by CAP (GE HealthCare). The procedure described above was followed but, in this case, serial dilutions of each sample (not less than 10) were incubated at RT for 2 h with the patient's sera (1:1, vol:vol), previously diluted in PBST, and 50 μL were added to each well. After 2 h of incubation at RT, the plate was washed three times and 50 μL of HRP-conjugated rabbit anti-human IgE (DakoCytomation, Glostrup, Denmark), diluted 1:1000 in PBST 0.05%, were added per well and incubated for 1 h at RT. Then the plate was washed three times and the tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Waltham, MA). Finally, the plate was washed four times and TMB was used as substrate. The reaction was stopped with 0.5 M sulfuric acid and the absorbance was measured at 450 nm. A negative control without serum (native protein in PBST) and positive controls (sera diluted in PBST 0.05%) were included in each plate.

Both IgG- and IgE-binding results were statistically processed. A non-linear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoid curve of inhibition dose-response with variable slope, from which the IC_{50} (the protein concentration that binds 50% of either IgG or seric IgE) was obtained with the program GraphPad PRISM 4 for Windows (GraphPad software, San Diego, CA). The IgG- and IgE-capacities of the digests were expressed as percentages of the IC_{50} of the intact protein (as means ± standard errors for $n = 3$). Significant differences ($p < 0.05$) were evaluated by one-way analysis of variance.

3. Results and discussion

3.1. Effect of the pH on the gastric digestion of LYS in vitro

Fig. 1 shows the SDS–PAGE pattern of LYS digested with pepsin at an E:S of 1:20 (wt/wt), after different hydrolysis times at different pHs, in order to simulate different conditions in the stomach, from the lowest values typical of a fasted state, to higher values characteristic of a fed state or of immature stomach functions. Hydrolysis of the protein only occurred at pH 1.2 and 2.0, with the formation of degradation products of less than 6 kDa that resisted pepsin action for at least 2 h of digestion. Interferences arising from the autodigestion of pepsin were also assessed, but no additional bands were detected in the electrophoretic separations at this enzyme concentration.

LYS had disappeared before 60 min of digestion at pH 1.2, while there still was unhydrolysed protein after 120 min at pH 2.0, a pH that prevails in the fasted stage of the stomach of healthy adults. The presence of a partially folded intermediate of LYS, characterised by a significant secondary structure, exposure of non-polar clusters and a disrupted tertiary structure, has been reported at very low pH values (1.5) (Polverino de Laureto, Frare, Gottardo, Van Dael, & Fontana, 2002). This increased flexibility could be responsible for its increased susceptibility to digestion at pH 1.2. The pH had a very important effect on LYS hydrolysis, compared to pepsin, which exhibits its optimum activity over a broad pH range, between 1.2 and 3.5. At pH values equal to or higher than 3.2, there was no detectable hydrolysis of the protein, even after 120 min of digestion (Fig. 1). The pH in the stomach of infants up to 2 months old is 3.0–4.0 and it can increase after food intake to values above 6.0 (Dupont et al., 2010).

While it is generally recognised that LYS is resistant to pepsin action (Polverino de Laureto et al., 2002), there is not much information on the proteolytic susceptibility of this protein and some discrepancies exist. Thus, Mine et al. (2004) reported its complete hydrolysis after 60 min of treatment at pH 1.0 and an E:S of 1:25 (wt:wt), while Fu, Abbott, and Hatzos (2002) claimed that it resisted more than 60 min at pH 1.2, at an E:S of 13:1 (wt:wt). Ibrahim et al. (2005) found that 40% of the original LYS was hydrolysed after 120 min of digestion at an E:S of 1:50 (wt:wt) and pH 4.0 (conditions that mimicked the infant stomach), giving three peptides with molecular masses of 7365, 5444 and 4317 kDa with a

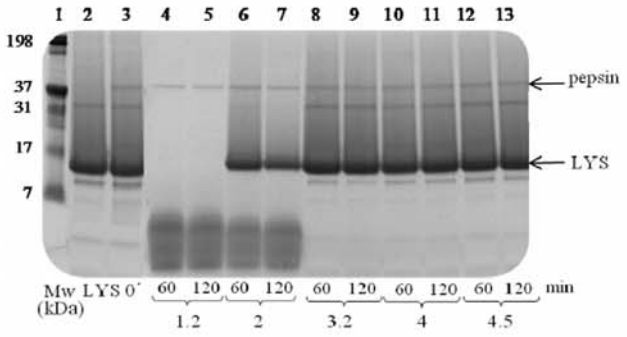

Fig. 1. SDS–PAGE analysis of LYS digested with pepsin, at a pepsin:protein ratio of 1:20 (w:w), at different pHs and hydrolysis times. Lane 1: molecular mass markers; lane 2: LYS; lanes 3, 4 and 5: LYS digested at pH 1.2 for 0, 60 and 120 min; lanes 6 and 7: LYS digested at pH 2.0 for 60 and 120 min; lanes 8 and 9: LYS digested at pH 3.2 for 60 and 120 min; lanes 10 and 11: LYS digested at pH 4.0 for 60 and 120 min; lanes 12 and 13: LYS digested at pH 4.5 for 60 and 120 min.

potent bactericidal activity. According to these authors, this could suggest the important biological role of pepsin hydrolysis of LYS from human milk as a defence system in the stomach of the newborn. However, under our digestion conditions, LYS was completely resistant to pepsin at pH values of 4.0 and 4.5 (Fig. 1).

3.2. Effect of the presence of PC on the gastric digestion of LYS in vitro

Fig. 2 compares the RP-HPLC pattern of *in vitro* gastric digestions performed for 60 min, at pH 2.0, in the absence and presence of PC, a physiological surfactant secreted by the gastric mucosa and also present in the bile. It should be mentioned that, regardless of the addition of PC, there were signs of protein precipitation on adjustment of the gastric digests to pH to 7.0 to irreversibly inactivate pepsin before the RP-HPLC analyses. The precipitate was removed by centrifugation and it was identified by RP-HPLC as unhydrolysed LYS. While residual LYS precipitated from the gastric digests at pH 7.0 solubilisation experiments demonstrated that the intact protein was fully soluble in SGF at different pHs up to 7.7, as well as in 10 mM phosphate buffer at pH 7.0 (results not shown).

As shown in Fig. 2, 6.3 mM PC (an 18:1 M ratio of PC:protein) did not have an important effect on the susceptibility of LYS to hydrolysis by pepsin, although it slightly increased LYS resistance to digestion, decreasing the presence of degradation products, without signs of changes in the fragmentation pattern (Fig. 2b and c). A similar behaviour has been reported for other proteins,

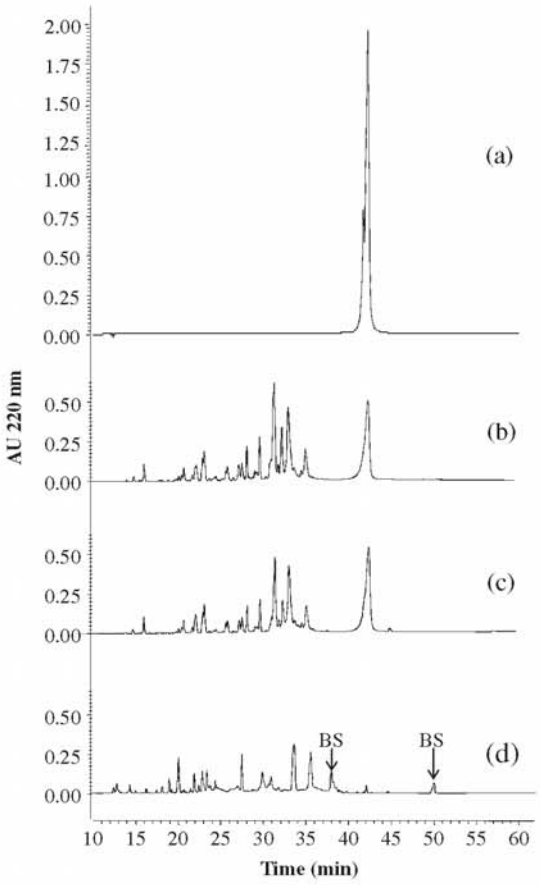

Fig. 2. RP-HPLC patterns of LYS (a) and its digests under different conditions (b–d). *In-vitro* gastric digestions at pH 2.0, for 60 min, in the absence (b); and presence (c) of phosphatidylcholine (PC); and *in-vitro* duodenal digest of (b) for 30 min (d). BS: bile salts.

such as LA, whose interaction with PC was shown to retard its proteolysis during gastric digestion, a result that was attributed to the partial penetration of LA into PC vesicles (Moreno et al., 2005b). In fact, when LA was digested with pepsin in the absence and presence of PC, the protective effect of PC on LA proteolysis was confirmed (Fig. 3). Thus, in agreement with the results of Moreno et al. (2005b), in the absence of PC, LA was rapidly hydrolysed by pepsin but, when PC was present, there was intact protein remaining after 5 and 15 min. On the other hand, pepsin hydrolysis of other proteins, such as the 2S albumin from Brazil nut (Moreno et al., 2005b, β-Lactoglobulin LG) (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Mandalari, Mackie, Rigby, Wickham, & Mills, 2009), or ovalbumin (OVA) (Martos et al., 2010) is not affected by the presence of PC in SGF at pH 2.5 or 2.0.

Hen egg white LYS and bovine LA are homologous to each other as they share similar primary structures, but their unfolding profiles and the stabilities of their native conformations are very different (Cawthern, Permyakov, & Berliner, 1996; Polverino de Laureto et al., 2002). The biological functions of LYS, such as its antimicrobial and immunomodulating properties, have been attributed to its ability to interact with membrane component phospholipids and to penetrate into lipid bilayers, aspects which have been broadly studied (Gorbenko, Loffe, & Kinnunen, 2007; Yuan et al., 2007). Similarly, the interaction of LA with phospholipid membranes and vesicles is of interest because membranes are implicated in the protein folding behaviour and in the ability of LA to regulate lactose synthesis (Cawthern et al., 1996).

The structural features of LYS and LA in the absence and presence of PC were studied by CD and fluorescence. The far-UV CD spectra of LYS (Fig. 4a) showed that the secondary structure of LYS in solution was the same at pH 2.0 and 7.0 and that it was not altered in the presence of PC. These results agree with Witoonsaridsilp, Panyarachun, Sarisuta, and Müller-Goymann (2010), who reported that the secondary structure of LYS entrapped in non-charged and negatively-charged liposomes, with various lipid compositions, does not significantly differ from that in buffer solutions at different pH values. The near-UV CD spectrum of LYS at pH 2.0 was typical of this protein, which does not change with respect to the neutral pH condition (Fig. 4b) (Polverino de Laureto et al., 2002). However, in the presence of PC at pH 7.0, a reduction in the signals in the near-UV region was patent, which suggested a disruption of the tertiary structure.

The far-UV spectrum of LA at neutral pH displayed two minima of ellipticity near 208 and 220 nm (Fig. 4c). Comparatively, at pH 2.0, it showed, approximately, 0.01% losses of alpha helix and beta strand. However, the near-UV spectra showed that the intensity of the dichroic signals in the 250–300 nm region was very low at pH 2.0, in comparison with pH 7.0 (Fig. 4d). This is in agreement with previous reports that indicated that, at acidic pH, LA maintains a native-like content of α-helical structure, but its tertiary structure is almost completely disrupted, due to the formation of a partially folded state named molten globule (Moreno et al., 2005b; Polverino de Laureto et al., 2002). The enhanced flexibility or local unfolding of LA over LYS at acidic pH probably favours cleavage of the former by pepsin, while LYS is highly resistant.

Intrinsic fluorescence emission spectra of LYS at pH 2.0 and 7.0 are shown in Fig. 5a. The presence of six tryptophan residues in LYS, with different spectral contributions because of their different polar environments, results in relatively broad fluorescence spectra (Gorbenko et al., 2007). The lower intensity at acidic pH can be attributed to a quenching effect exerted by protonated acidic groups. Upon titration with PC at pH 2.0, the wavelength of maximum emission (λ_{max}) on excitation at 280 nm changed from 343 nm to 340 nm. Since the transfer of Trp into an environment with a lower polarity usually coincides with a blue shift of the λ_{max}, this suggests that mixing of LYS with PC modified the local

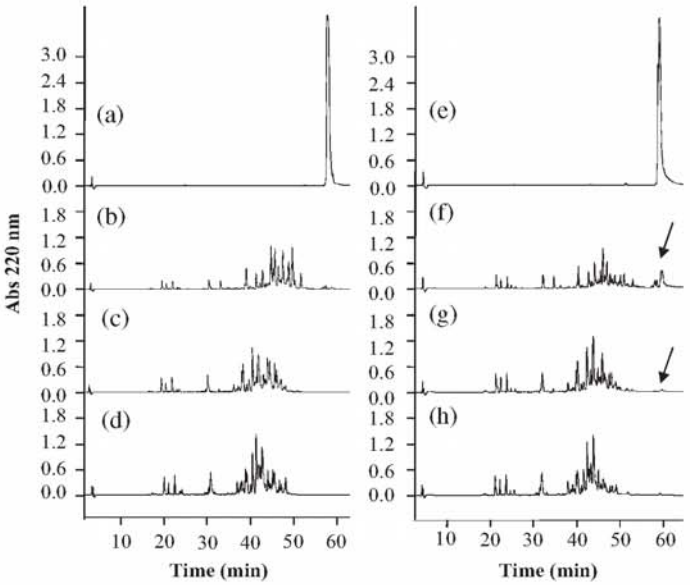

Fig. 3. RP-HPLC patterns of LA (a, e) and its pepsin digests obtained at pH 2.0 in the absence (a–d); and presence (e–h) of phosphatidylcholine (PC); at different hydrolysis times: 0 min (a, e), 5 min (b, f), 15 min (c, g), and 30 min (d, h).

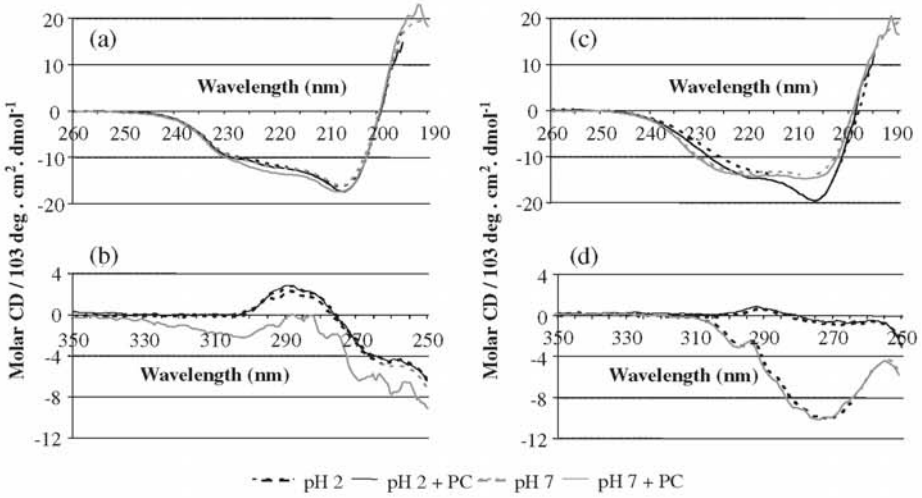

Fig. 4. Circular dichroism spectra in the far (a, c) and near (b, d) UV region of LYS (a, b) and LA (c, d), at pH 2.0 and 7.0 in the absence and presence of phosphatidylcholine (PC).

environment of the protein making it more hydrophobic, possibly through membrane binding. In fact, a blue shift in the spectrum of LYS has been attributed to a shielding effect from water arising from the penetration of the protein into a phospholipid bilayer (Yuan et al., 2007). When different amounts of PC were titrated into a fixed concentration of LYS at acidic pH the fluorescence intensity decreased regularly (Fig. 5c), suggesting an interaction between the phospholipid and the protein that changed the accessibility of Trp residues to the solvent. On the other hand, at neutral pH, fluorescence intensity hardly changed on PC addition.

Regarding bovine LA, the longer wavelength of the λ_{max} and the higher fluorescence intensity of the spectrum at pH 2.0, as compared to pH 7.0, supported that the Trp in the molten globule are more accessible to the solvent than in native LA (Svensson et al., 1999) (Fig. 5b). In this case, titration experiments showed that the fluorescence intensity progressively changed with the concentration of PC, decreasing at pH 2.0 and increasing at pH 7.0 (Fig. 5b and d). This suggests that, at pH 2.0, there is a partial insertion of Trp into the apolar phase of the lipid bilayer, while at pH 7.0, association leads to an increase in Trp accessibility. In fact, at acidic pH, LA behaves as a membrane intrinsic protein and penetrates into PC vesicles. Below its isoelectric point (4.8), hydrophobic forces dominate the interaction of LA and PC, with binding being probably reinforced by the unfolding of the protein that increases surface hydrophobicity (Cawthern et al., 1996; Moreno et al., 2005b). Above its isoelectric point, the interaction with the zwitterionic phospholipid is mainly electrostatic, which induces a loose association with the outer surface of the vesicles (Moreno et al., 2005b). Deep insertion of proteins into membranes has been reported to prevent their degradation on incubation with

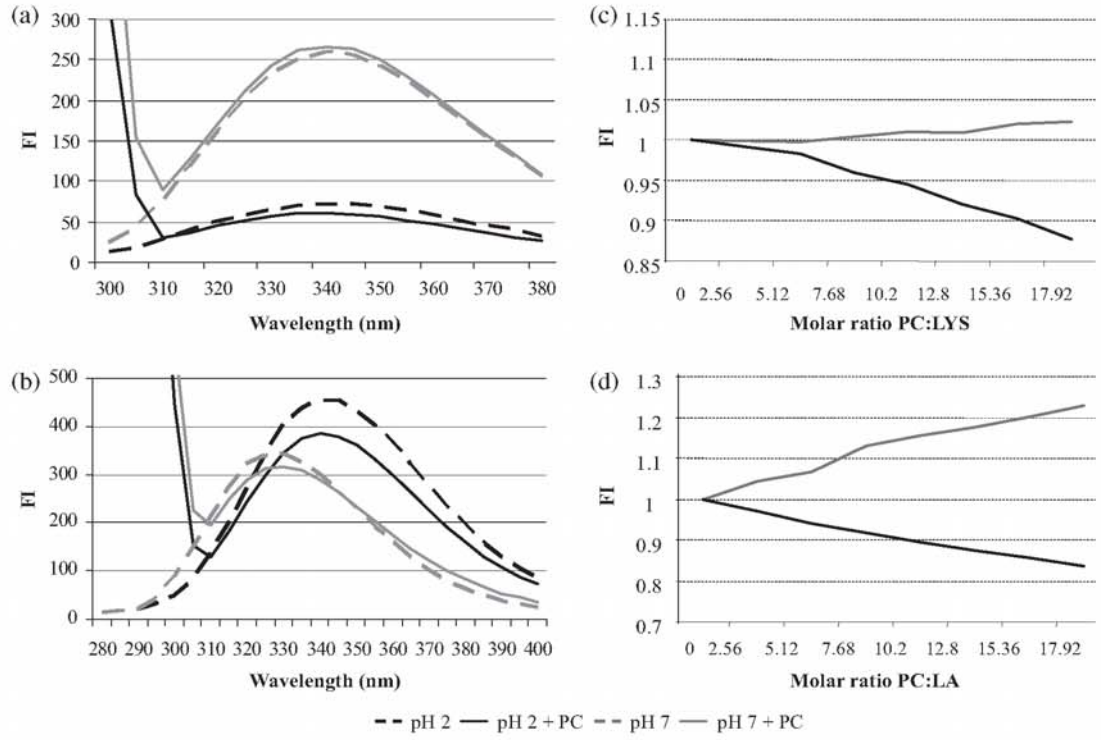

Fig. 5. Fluorescence spectra of LYS (a) and LA (b), at pH 2.0 and 7.0, in the absence and presence of phosphatidylcholine (PC), and corrected LYS (c) and LA (d) titration curves with PC at pH 2.0 and pH 7.0.

proteases, while membrane association, without losing protein integrity, may make them more sensitive to proteolytic degradation (Mogensen et al., 2007).

While LA attains a molten globule estate at pH 2.0, LYS remains native. Nevertheless, LYS could still interact with zwitterionic phospholipids, such as PC, through hydrophobic and polar interactions that could lead to LYS association to PC films. In fact, it has been reported that LYS could be entrapped in non-charged PC liposomes (Witoonsaridsilp et al., 2010). However, penetration is difficult once adsorption has occurred, because LYS is excluded from interacting with the hydrophobic portion of the lipid below the chain transition temperature of PC, which is approximately 41 °C (Mudgil, Torres, & Millar, 2006). On the other hand, LYS, with an isoelectric point near 11, is a highly electropositive protein, that can easily penetrate anionic phospholipid vesicles, such as those formed by phosphatidylserine or phosphatidylglycerol. In this case, LYS binding to anionic vesicles is governed by electrostatic effects that lead to LYS penetration into the lipid phase (Mudgil et al., 2006; Witoonsaridsilp et al., 2010).

3.3. Effect of the presence of BS and PC on the Duodenal digestion of LYS in vitro

The gastric digests of LYS produced after 60 min at pH 2.0, with and without the addition of PC, once adjusted to pH 7.0 and centrifuged, were subjected to an *in vitro* duodenal digestion for 30 min with trypsin and chymotrypsin in the presence of BS. Under those conditions, LYS completely disappeared (Fig. 2d), despite, according to the literature, trypsin and chymotrypsin hardly hydrolysing the protein (During, Porsch, Mahn, Brinkmann, & Gieffers, 1999), or just hydrolysing it partially after overnight incubation at 37 °C (Mine et al., 2004).

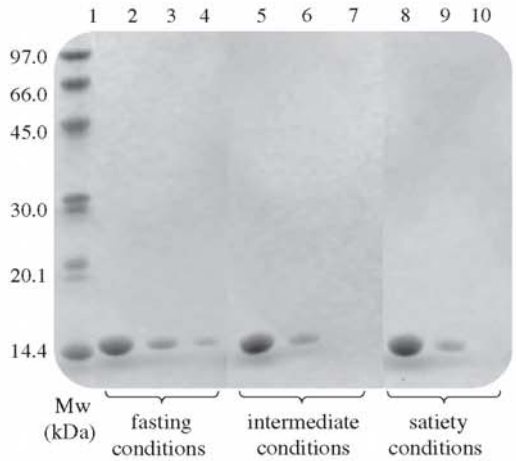

Fig. 6. SDS–PAGE analysis of LYS soluble in 7.6 mM $CaCl_2$ and 20.3 mM Bis–Tris mimicking duodenal conditions typical of a fasted (2, 3, 4); intermediate (5, 6, 7) and fed (8, 9, 10) state. Lane 1: molecular mass markers; lane 2: LYS at pH 7.0; lane 3: LYS at pH 7.0 with 3 mM of each BS and 2.4 mM PC; lane 4: LYS at pH 7.0 with 3 mM of each BS; lane 5: LYS at pH 6.5; lane 6: LYS at pH 6.5 with 6 mM of each BS and 6.5 mM PC; lane 7: LYS at pH 6.5 with 6 mM of each BS; lane 8: LYS at pH 6.0; lane 9: LYS at pH 6.0, with 9 mM of each BS and 7.2 mM PC; lane 10: LYS at pH 6.0 with 9 mM of each BS. BS: bile salts.

To elucidate whether the presence of BS could have an effect on LYS solubility, we incubated the intact protein with three different duodenal digestion media, typical of a fasted state, an intermediate state similar to the duodenal mixture we used for digestion, and a fed state, but without proteases and lipases. Fig. 6 shows the effect

on LYS solubility of the different simulated small intestine conditions.

LYS was fully soluble in 7.6 mM $CaCl_2$ and 20.3 mM Bis–Tris at pH 6.0, 6.5, and 7.0, but it precipitated in the presence of BS. The concentration of amphiphilic bile components in the upper intestine, including BS and PC, increases after a meal, while the pH decreases (Kostewicz et al., 2002). As shown in Fig. 6, the higher the bile salt concentration the lower the amount of soluble LYS. This indicated that the disappearance of the protein could not be totally attributed to its hydrolysis by duodenal enzymes but, to some extent, to its precipitation with BS in the duodenal medium. It should be noted that the presence of PC partially avoided LYS precipitation, suggesting a positive effect on solubilisation of the mixed bile salt-PC micelles present in the duodenal medium (Mandalari et al., 2009). It should be noted that the BS concentration used was always over the critical micelle concentration, 3.5 mM (Kaukonen et al., 2004).

Although, according to Burnett et al. (2002), BS exert a solubilising effect, removing proteins adsorbed to emulsions and favouring their resolubilisation in aqueous phases, our results show that it is likely that LYS precipitates in the duodenum at pH values, BS and PC concentrations representative of a fed state and, to a lesser extent, of a fasted state. In fact, when LYS is administered orally to human patients (as used for the treatment of chronic sinusitis and to promote expectoration in the case of respiratory disease), food intake negatively affects the uptake of the enzyme, with the maximum levels detected being almost 10-fold lower than those after an identical dose taken after an overnight fast (Hashida, Ishikawa, Nakamichi, & Sekino, 2002).

Insolubilisation of LYS in the presence of BS could impair its hydrolysis by pancreatic enzymes, affecting its presence in the intestinal tract. Despite its molecular weight, LYS can be effectively absorbed and its uptake occurs preferentially in the upper intestine (Takano, Koyama, Nishikawa, Murakami, & Yumoto, 2004). Absorption of the intact protein could be particularly relevant in infants, whose immature gastrointestinal epithelium allows more proteins to cross the barrier into the circulatory system. Furthermore, while LYS precipitation could play a role in the uptake of LYS by intestinal brush border membranes and its presentation to the immune system, it may positively enhance the proportion of antimicrobial agents that can play a defensive role in lower parts of the intestine.

3.4. Effect of digestion on IgG and IgE binding

The IgG and IgE-binding properties of the gastric digests obtained after 60 min were examined by inhibition ELISA. The IgG-binding was estimated with a polyclonal commercial antibody against LYS raised in rabbit, while for IgE-binding we used sera of patients allergic to egg with proved immunoreactivity against LYS.

The digests exhibited a substantial residual antigenicity both against IgG and IgE (Fig. 7a and b), which could only be somewhat attributed to the presence of residual LYS, since, as already mentioned, LYS partially precipitated (around 40–45% as estimated by RP-HPLC) when the pH was adjusted to 7.0 to inactivate pepsin. This indicated that some of the proteolysis products retained a noticeable reactivity against IgG and IgE and that, in fact, the reactivity of the digests was comparatively higher than that of the intact protein. The highest IgG-binding was detected in the gastric digests obtained in the presence of PC ($p < 0.05$), which also contained more intact protein than those produced in the absence of PC as showed above (Fig. 3). Regarding IgE-binding, as expected, the responses were shown to vary depending on the patient's individual susceptibility. In this case, there were no differences between the hydrolysates obtained with or without PC. Overall, these results show that *in vitro* gastric digestion of LYS led to the formation of peptides which carried an important epitope load and thus, the potential to be allergenic.

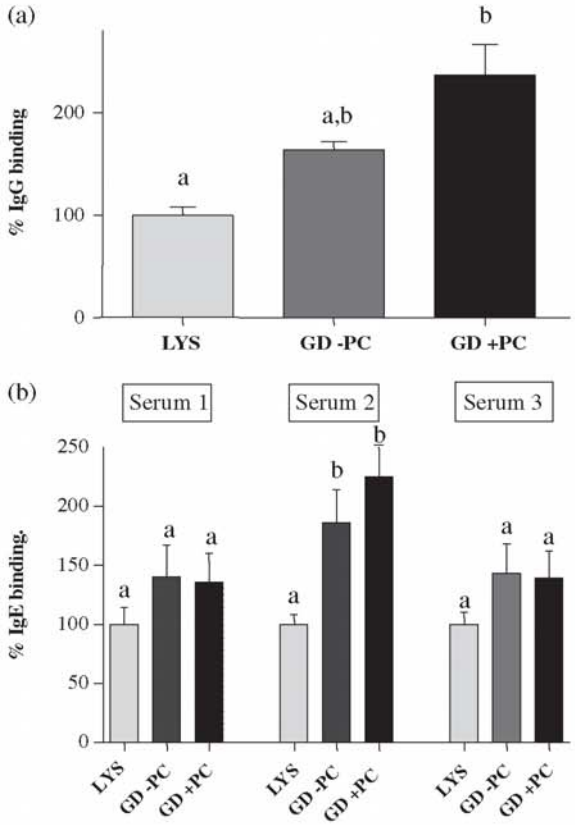

Fig. 7. Inhibition ELISA response against rabbit polyclonal anti-LYS IgG (a) and human IgE (b) of LYS and its pepsin digests obtained at pH 2.0 in the absence (−PC) and presence of phosphatidylcholine (+PC) for 60 min. Human sera were IgE >100 kU/L. The IgG- and IgE-binding capacities of the digests were expressed as percentages of the IC_{50} of the intact protein. Significant differences ($p < 0.05$) were evaluated by one-way analysis of variance. Different letters above the bars indicate significant differences ($p < 0.05$). Error bars correspond to the mean ± standard error ($n = 3$).

4. Conclusions

Digestion of LYS with pepsin was conducted at an enzyme: substrate ratio of 1:20 (wt/wt) (172 U/mg), considered as a physiological ratio, under different pHs that simulated various conditions in the stomach. Hydrolysis of the protein only occurred at pH 1.2 and 2.0, typical of a fasted state, with the formation of degradation products of less than 4–5 kDa that resisted pepsin action for at least 2 h of digestion. At pH values equal or higher than 3.2, typical of a fed state or of immature stomach functions, there was no detectable hydrolysis of the protein, even after 120 min of digestion. The presence of PC, a physiological surfactant, slightly increased LYS resistance to digestion, decreasing the presence of degradation products, and a similar behaviour was found for its homologous protein LA. At acidic pH, the enhanced flexibility or local unfolding of LA over LYS, as determined by circular dichroism spectroscopy probably favoured the cleavage of the former by pepsin, while LYS was more resistant. Intrinsic fluorescence emission spectra of LYS and LA at pH 2.0, on addition of PC, suggested that the local environment of the proteins became more hydrophobic, possibly through membrane binding. It is, therefore, likely that,

at acidic pH, both proteins interact with PC, leading to their association with PC films.

IgE-binding experiments using sera of patients allergic to egg showed that *in vitro* gastric digestion of LYS led to the formation of peptides which carried an important epitope load and could have the potential to be allergenic. The identification of these peptides and their resistance to hydrolysis by pancreatic proteinases are currently being addressed. On the other hand, our results show that unhydrolysed LYS precipitated at pH values, and BS and PC concentrations representative of a duodenal fed state (characterised by high BS and PC concentrations and low pH) and, to a lesser extent, of a fasted state. LYS precipitation in the presence of BS could impair its hydrolysis by pancreatic enzymes, either affecting the amount of immunoreactive protein that is absorbed or the proportion of antimicrobial agents that can play a defensive role in lower parts of the intestine.

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References

Barbana, C., Pérez, M. D., Sánchez, L., Dalgalarrondo, M., Chobert, J. M., & Haertlé, T. (2006). Interaction of bovine α-lactalbumin with fatty acids as determined by partition equilibrium and fluorescence spectroscopy. *International Dairy Journal, 16*, 18–25.

Burnett, G. R., Wickham, M., Fillery-Travis, A., Robertson, J. A., Belton, P. S., Gilbert, S. M., et al. (2002). Interaction between protein allergens and model gastric emulsions. *Biochemical Society Transactions, 30*, 916–918.

Cawthern, K. M., Permyakov, E., & Berliner, L. J. (1996). Membrane-bound states of alpha-lactalbumin: Implications for the protein stability and conformation. *Protein Science, 5*, 1394–1405.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Leonil, J., Faulks, R. M., et al. (2010). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Molecular Nutrition and Food Research, 54*, 767–780.

During, K., Porsch, P., Mahn, A., Brinkmann, O., & Gieffers, W. (1999). The non-enzymatic microbicidal activity of lysozymes. *FEBS Letters, 449*, 93–100.

Fox, P. F., & Kelly, A. L. (2006). Indigenous enzymes in milk: Overview and historical aspects - Part II. *International Dairy Journal, 16*, 517–532.

Fu, T.-J., Abbott, U. R., & Hatzos, C. (2002). Digestibility of food allergens and non allergenic proteins in simulated gastric fluid and simulated intestinal fluid – A comparative study. *Journal of Agricultural and Food Chemistry, 50*, 7154–7160.

Gorbenko, G. P., Loffe, V. M., & Kinnunen, P. K. J. (2007). Binding of lysozyme to phospholipid bilayers: Evidence for protein aggregation upon membrane association. *Biophysical Journal, 93*, 140–153.

Hashida, S., Ishikawa, E., Nakamichi, N., & Sekino, H. (2002). Concentration of egg white lysozyme in the serum of healthy subjects after oral administration. *Clinical and Experimental Pharmacology and Physiology, 29*, 79–83.

Iaconelli, A., Fiorentini, L., Bruschi, S., Rossi, F., Mingrone, G., & Piva, G. (2008). Absence of allergic reactions to egg white lysozyme additive in Grana Padano cheese. *Journal of the American College of Nutrition, 27*, 326–331.

Ibrahim, H. R., Inazaki, D., Abdou, A., Aoki, T., & Kim, M. (2005). Processing of lysozyme at distinct loops by pepsin: A novel action for generating multiple antimicrobial peptide motifs in the newborn stomach. *Biochimica et Biophysica Acta, 1726*, 102–114.

Kaukonen, A. M., Boyd, B. J., Porter, C. J. H., & Charman, W. N. (2004). Drug solubilisation behaviour during in vitro digestion of simple triglyceride lipid solution formulations. *Pharmaceutical Research, 21*, 245–253.

Kostewicz, E. S., Brauns, U., Becker, R., & Dressman, J. B. (2002). Forecasting the oral absorption behaviour of poorly soluble weak bases using solubility and dissolution studies in biorelevant media. *Pharmaceutical Research, 19*, 345–349.

Lesnierowski, G., & Kijowski, J. (2007). Lysozyme. In R. Huopalathi, R. López-Fandiño, M. Anton, & R. Schade (Eds.), *Bioactive egg compounds* (pp. 33–42). Berlin: Springer-Verlag.

López-Exposito, I., & Recio, I. (2006). Antibacterial activity of peptides and folding variants from milk proteins. *International Dairy Journal, 16*, 1294–1305.

Macierzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M., & Mackie, A. R. (2009). Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin. *Soft Matter, 5*, 538–550.

Mandalari, G., Mackie, A. M., Rigby, N. M., Wickham, M. S. J., & Mills, E. N. C. (2009). Physiological phosphatidylcholine protects bovine beta-lactoglobulin from simulated gastrointestinal proteolysis. *Molecular Nutrition and Food Research, 53*, 131–139.

Martos, G., Contreras, P., Molina, E., & López-Fandiño, R. (2010). Egg white ovalbumin digestion mimicking physiological conditions. *Journal of Agricultural and Food Chemistry, 58*, 5640–5648.

Mine, Y., Ma, F., & Lauriau, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *Journal of Agricultural and Food Chemistry, 52*, 1088–1094.

Mine, Y., & Yang, M. (2008). Recent advances in the understanding of egg allergens: Basic, industrial and clinical perspectives. *Journal of Agricultural and Food Chemistry, 56*, 4874–4900.

Mogensen, J. E., Ferreras, M., Wimmer, R., Petersen, S. V., Enghild, J. J., & Otzen, D. E. (2007). The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. *Biochemistry, 46*, 3356–3365.

Moreno, F. J. (2007). Gastrointestinal digestion of food allergens: Effect on their allergenicity. *Biomedicine and Pharmacotherapy, 61*, 50–60.

Moreno, F. J., Mackie, A. R., & Mills, E. C. N. (2005b). Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. *Journal of Agricultural and Food Chemistry, 53*, 9810–9816.

Moreno, F. J., Mellon, F. A., Wickman, M. S. J., Bottrill, A. R., & Mills, E. N. C. (2005a). Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro digestion. *FEBS Journal, 272*, 341–352.

Mudgil, P., Torres, M., & Millar, T. J. (2006). Adsorption of lysozyme to phospholipids and meibomian lipid monolayer films. *Colloids and Surfaces B, 48*, 128–137.

Pellegrini, A., Thomas, U., Bramaz, N., Klauser, S., Hunziker, P., & Von Fellneberg, R. (1997). Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *Journal of Applied Bacteriology, 82*, 372–378.

Polverino de Laureto, P., Frare, E., Gottardo, R., Van Dael, H., & Fontana, A. (2002). Partly folded status of members of the lysozyme/lactalbumin superfamily: A comparative study by circular dichroism spectroscopy and limited proteolysis. *Protein Science, 11*, 2932–2946.

Sobrino-López, A., & Martín-Belloso, O. (2008). Enhancing the lethal effect of high-intensity pulsed electric field in milk by antimicrobial compounds as combined hurdles. *Journal of Dairy Science, 91*, 1759–1768.

Svensson, M., Sabharwal, H., Håkansson, A., Mossberg, A. K., Lipniunas, P., Leffler, H., et al. (1999). Molecular characterisation of α-lactalbumin folding variants that induce apoptosis in tumor cells. *Journal of Biological Sciences, 274*, 6388–6396.

Takano, M., Koyama, Y., Nishikawa, H., Murakami, T., & Yumoto, R. (2004). Segment-selective absorption of lysozyme in the intestine. *European Journal of Pharmacology, 502*, 149–155.

Witoonsaridsilp, W., Panyarachun, B., Sarisuta, N., & Müller-Goymann, C. C. (2010). Influence of microenvironment and liposomal formulation on secondary structure and bilayer interaction of lysozyme. *Colloids and Surfaces B, 75*, 501–509.

Yuan, B., Xing, L. L., Zhang, Y. D., Lu, Y., Luo, Y. Y., Mai, Z. H., et al. (2007). Penetration and saturation of lysozyme in phospholipids bilayers. *Journal of Physical Chemistry B, 111*, 6151–6155.

III

Mol. Nutr. Food Res. (enviado)

Immunoreactivity of hen egg allergens: influence on *in-vitro* gastrointestinal digestion of the presence of other egg white proteins and of egg yolk.

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Molecular Nutrition and Food Research

Immunoreactivity of hen egg allergens: influence on *in-vitro* gastrointestinal digestion of the presence of other egg white proteins and of egg yolk.

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Abbreviations: TBS, Tris buffered saline; **TBST**, Tris buffered saline containing 0.05% Tween 20; **SGF,** Simulated gastric fluid; **OM,** ovomucoid; **OVA,** ovalbumin; **OVT,** ovotransferrin; **LYS,** lysozyme; **PBST,** phosphate buffered saline containing 0.05% Tween 20; **OvoI,** ovoinhibitor

Keywords: clusterin / egg white allergens / gastrointestinal digestion / IgE-binding / ovoinhibitor

ABSTRACT

Hen egg white comprises a complex mixture of proteins which greatly differ in their physicochemical characteristics and relative abundance. We aimed to identify potential undiscovered egg allergens within the egg white proteome and investigated the existence of matrix effects on the proteolytic stability and resultant IgE-binding of the major allergenic proteins. Two minor egg white proteins separated by 2D electrophoresis, and tentatively identified as ovoinhibitor and clusterin, were found to react with serum IgE from egg-allergic patients. Lysozyme (LYS) also showed an outstanding reaction against IgE. Egg white exhibited an important residual immunoreactivity after gastrointestinal digestion due to the presence of intact ovalbumin (OVA) and LYS, as well as of many IgE- binding peptides that derived from OVA, as assessed by peptide mass fingerprinting. In fact, OVA was more resistant to gastrointestinal digestion as part of the egg white protein mixture as compared with the isolated protein. The presence of egg yolk slightly increased the susceptibility to hydrolysis of egg white proteins and abrogated bile salt-induced precipitation of LYS in the duodenal medium. However, the resultant immunoreactivity against IgE of egg white proteins after *in vitro* digestion was not significantly modified by the presence of yolk components.

1 INTRODUCTION

Hen eggs are an important and inexpensive supply of high quality proteins in the human diet, but also the second most frequent source of allergic reactions, particularly in children [1]. The main allergens: ovomucoid (OM), ovalbumin (OVA), ovotransferrin (OVT) and lysozyme (LYS), named Gal d 1-4, respectively, are contained in the egg white, while two yolk-specific allergens have been reported so far: α-livetin (Gal d 5) and protein YGP42 (Gal d 6) [2, 3]. Egg proteins are very heterogeneous with respect to their molecular masses (12.7-8000 kDa) and pI values (4-11) [4]. Furthermore, they are present in very different concentrations, with OVA, OM and OVT comprising approximately 75% of the total proteins. Recent works have considerably widened our knowledge of the egg white proteome [5, 6], characterising several minor proteins, but their potential contribution to egg allergy has not been explored.

In order to elicit an IgE response, an allergen must be able to cross the intestinal barrier, retaining sufficient structural integrity to interact with the immune system. Therefore, a major characteristic of many food allergens is their resistance to gastric digestion [7]. However, several food allergens have been identified that are sensitive to these processes; while many non-allergenic proteins are as resistant to degradation as allergenic proteins [8]. We have previously employed an *in vitro* digestion model that takes into account the physiological concentrations of enzymes and surfactants present in the stomach and intestine [9] to assess OVA, OM and LYS immunoreactivity after digestion [10-12]. The results obtained underline the importance of taking into account the complex factors that play a role in physiological digestion and of evaluating the

ability of the fragments generated upon digestion to retain biologically relevant IgE epitopes.

An aspect of food allergens that remains to be elucidated is the influence of the food matrix on the immune responses [13]. The food matrix, composed of proteins, carbohydrates, lipids and micronutrients, has been suggested to affect the allergenic properties of proteins by providing adjuvant stimuli to the specialized gut mucosal immune system or by protecting them from digestion [14]. In fact, the stability of proteins to digestion may be altered in the presence of various components that form part of the food matrix. Thus, several studies have demonstrated that the presence of soluble polysaccharides reduces protein digestibility [15]. Similarly, many food proteins can interact with lipids to form emulsions and other structures, exhibiting a different susceptibility to digestion [16]. Furthermore, the biological activity of certain proteins may promote the conditions necessary for specific sensitization or elicitation of the immune response. For instance, in the case of proteins with protease-inhibitory activity, a major peanut allergen, Ara h2 was found to act as a trypsin inhibitor that, in addition to being more resistant to trypsin digestion itself, was found to protect Ara h1, a second major peanut allergen, from degradation by trypsin [17]. In this respect, it is important to note that OM is an important trypsin inhibitor, and that pepsin digested OM retains its trypsin inhibitor activity, what helps ovomucoid and its pepsin digestion fragments to be stable in the small intestine [18].

In this paper we studied the IgE reactivity of egg white proteins with the double objective to find potential undiscovered allergens among the minor proteins and to evaluate some general matrix effects on their proteolytic stability and resultant IgE-

binding. Susceptibility to *in vitro* digestion assays was examined in the context of mixtures of compounds (whole egg white or egg white and yolk) and compared with previous results on isolated proteins. We also assessed the immunoreactivity of egg white proteins after *in vitro* digestion with a view to find resistant peptides which preserved IgE epitopes.

2 MATERIALS AND METHODS

2.1 Reagents and materials. All reagents and materials were purchased from Bio-Rad (Richmond, CA, USA) unless otherwise specified. For in vitro digestion, chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Eggs came from organic-crop fed poultry and were purchased from a local supermarket.

Different serum pools from children with clinical allergic symptoms to egg were used at a dilution 1:200 in pH 7.6 tris buffered saline (TBS) containing 0.05% Tween 20 (TBST). The sera were collected from the Maternal and Child Gregorio Marañon Hospital (Madrid, Spain). The patients' specific seric IgE levels were determined by CAP (GE HealthCare).

2.2 In vitro gastric and duodenal digestion. Egg whites and yolks were either separated or mixed with a magnetic stirrer at the weight ratio found in the egg and their protein concentration was measured by the Bradford assay [19]. Solutions in simulated gastric fluid (SGF, 35 mM NaCl at pH 2) were prepared at a concentration of 6 mg protein/mL. The corresponding substrates in SGF were preheated for 15 min at 37 ºC

before incubation with porcine pepsin (EC 3.4.23.1, 3440 units/mg) at an enzyme:protein ratio of 1:20, w/w (172 units/mg). The reactions were stopped after 60 min by raising pH to 7.0 with $NaHCO_3$.

Duodenal digestions were performed by using, as the starting substrate, the 60 min gastric digests adjusted to pH 7 with the addition of: 1M $CaCl_2$, 0.25M Bis-Tris pH 6.5 and a 0.125M bile salts mixture containing equimolar quantities of sodium taurocholate and glycodeoxycholic acid. After preheating at 37 ºC for 15 min, aqueous solutions of trypsin (EC 232-650-8, 10100 BAEE units/mg protein), α-chymotrypsin (EC 232-671-2; 55 units/mg protein), porcine pancreatic lipase (EC 232-619-9) and colipase (EC 259-490-1) were added to the duodenal mix. The final composition of the mixture was: 6.15 mM of each bile salt, 20.3 mM Bis-Tris, 7.6 mM $CaCl_2$; and the enzymes referred to the quantity of protein were: 40 units/mg trypsin, 0.5 units/mg α-chymotrypsin, 28.9 units/mg lipase and colipase (enzyme:protein ratio 1:895 w/w). The reactions were stopped after 30 min with trypsin-chymotrypsin inhibitor following the manufacturer indications. The final protein concentration of all samples was adjusted to 4 mg/mL.

Aliquots of egg gastric and gastroduodenal digests were lyophilized for their analysis by SDS and 2DE and immunoblotting.

2.3 2DE. To improve the relative amount of minor proteins, the egg white (1:1 in PBS) was first treated with the ProteoMiner™ enrichment kit following the instructions of the manufacturer. Excess of salts and other interferents were removed from the eluates using the ReadyPrep™ 2-D Cleanup kit. 140 µL of the eluate were

mixed with 60 µL of rehydration buffer (thiourea was added to the buffer to make it 2M). In the case of lyophilized gastroduodenal digests, enrichment and cleaning steps were omitted. Powder (62 mg protein) was resuspended in 3 mL of water and 10 µL were diluted 1/20 in rehydration buffer

200 µL of sample were loaded on an immobilized pH gradient (IPG) strip pH 4-7 and a rehydration step was carried out in a Protean® IEF Cell at 50V overnight. Isoelectric focusing was performed as follows: a lineal ramp to 250V in 15 min, then up to 8000V in 2h 30min; this voltage was maintained until accumulating 20000Vh and then a rapid ramp down to 500V, voltage held until reaching 30000V·h. The total separation time was about 5h 30min. The IPG strip was reduced and alkylated for 10 and 15 min with equilibration buffers I and II (Bio-Rad) containing DTT and iodoacetamide, respectively. Then it was washed in XT-MES running buffer and placed in the slot for IPG strip of a Bis-tris 12% polyacrylamide gel. Agarose was used to link both dimensions. The second dimension was performed at 150 V in a Criterion™ cell using the XT-MES running buffer. SYPRO® Ruby fluorescent dye was used following the manufacturer's instructions for protein detection in the undigested egg white sample. Egg white gastroduodenal digest sample was detected by silver staining following Mortz et al. [20].

2.4 SDS-PAGE. Samples were diluted 1:4 (final protein concentration 1 mg/mL) in a buffer containing 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-Mercaptoethanol and 0.0025% (w/v) bromophenol blue. Then, they were heated for 5 min at 95 ºC and 40 µL were loaded on a 12% Bis-tris polyacrylamide

gel (Criterion™ XT). Electrophoretic separation was carried out at 150 V in a criterion cell using a XT-MES running buffer. Gels were stained with Coomassie Blue G-250.

2.5 Western blotting. Mono- and bidimensional unstained gels were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min. Proteins were electroblotted into a nitrocellulose membrane using a Trans-Blot® SD apparatus for 30 min at 18V.

For detection with commercial polyclonal antibodies, the nitrocellulose membranes were incubated in TBST containing 1% bovine serum albumin (TBST-1% BSA) overnight at 4ºC. Next day, OVA antibody (GeneTex, Irvine, CA, USA), rabbit anti chicken OM antibody (Immune Systems Ltd., Devon, United Kingdom) and LYS antibody (Abcam, Cambridge, UK), 1/30000, 1/10000 and 1/20000 diluted in TBST-0.1% BSA, respectively, were incubated with the membrane for 1h at room temperature. All incubations were followed by 5 washing steps with TBST of 5 min each. Finally, the membrane was developed with the chemiluminescent substrate (Novagen, Darmstadt, Germany). Image acquisition (exposure time 10 min) was performed using the VersaDoc Imaging System.

For detection with human sera, the membranes were incubated in TBST-1% BSA for 3h and dipped into a pool or sera from children with clinical allergic symptoms to egg at (1/40 diluted in TBST-0.1% BSA) for 24 h at 4ºC. Next, the membrane was incubated with biotin-conjugated anti-human-IgE antibody (Southern Biotech, Birmingham, AL, USA) 1/500 diluted in TBST-0.1% BSA, overnight at 4ºC. Finally, Horseradish peroxidase (HRP)-Streptavidin (DakoCytomation, Glostrup, Denmark)

diluted 1:3000 in TBST-0.1% BSA was added and incubated for 1 h. All incubations were followed by 5 washing steps with TBST of 5 min each. The chemiluminescent substrate Amersham™ ECL™ Prime (GE HealthCare, Uppsala, Sweden) was used and light emission detected for 5 min in the VersaDoc™ imaging system.

2.6 Mass spectrometry analyses of peptide bands and spots. Bands and spots of interest were manually excised from the corresponding gels using biopsy punches. Bands and spots selected for analysis were digested with trypsin according to Shevchenko et al. [21]. Peptide bands from the monodimensional gel were in-gel reduced and alkylated prior to trypsin digestion. After digestion, the supernatant was collected and spotted onto a MALDI target plate with α-cyano-4-hydroxycinnamic acid matrix for protein identification by peptide mass fingerprint. Analyses were performed in a 4700 Proteomics MALDI-TOF/TOF Analyzer (Perseptives Biosystems, Framingham, MA). SwissProt DB v. 57.15 with taxonomy restriction to Metazoa (Animals) was searched using MASCOT 2.2 (matrixscience.com) through the Global Protein Server v3.6 from ABSciex. Search parameters were carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; peptide mass tolerance 50 ppm and allowance for 1 missed trypsin cleavage site. Probability scores were greater than the score fixed by MASCOT as significant with P-value<0.05.

2.7 RP-HPLC. Samples were diluted to a protein concentration of 2 mg/mL, filtered through a 0.2 μm pore size and then separated in a Hi-Pore RP-318 (250 x 4.6 mm internal diameter) column (Bio-Rad) in a Waters 600 HPLC (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and UV detector. The digests were eluted by using 0.037% (v/v) trifluoroacetic acid in double-distilled water

as solvent A and 0.027% (v/v) trifluoroacetic acid in acetonitrile as solvent B, at 1mL/min, and detection was at 220 nm. The chromatographic method was that described by Quirós et al. [22]. Data were processed by using Empower 2 Software (Waters Corporation).

2.8 Inhibition ELISA. Immunoreactivity of egg white and yolk digests was quantified by inhibition ELISA as follows. Single wells of polystyrene microtiter plates were coated with undigested egg white and yolk samples at a protein concentration of 50 µg/mL in PBS, and incubated overnight at 4 °C. Plates were washed with PBST after each subsequent incubation step. Blocking was performed with PBS containing 2.5% Tween 20 for 3h at room temperature. Then, serial dilutions of each sample (not less than seven) were incubated during 2h with serum pools (1:1, v:v), and 50 µL were added to each well. After a further 2h of incubation, 50 µL of polyclonal rabbit anti-human IgE (Dako Denmark A/S, Glostrup, Denmark) 1:1000 diluted in PBST, were added per well and incubated for 1h. Next, 50 µL of polyclonal swine anti-rabbit immunoglobulins/HRP (Dako Denmark A/S) were added and incubated for another hour. The tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Walthman, MA, USA). Finally, tetramethyl benzidine was used as substrate. Reaction was stopped with 0.5 M sulphuric acid. Absorbance was measured at 450 nm. A negative control without serum (undigested egg white and yolk sample at 1mg/mL in PBST) and positive controls (serum pools diluted in PBS) were included in each plate.

A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal curve of inhibition dose-response with variable slope, from which the IC50 (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad PRISM 4 (www.graphpad.com).

3 RESULTS AND DISCUSSION

3.1 Immunoreactivity against IgE of egg white proteins

With the aim to identify potential allergens among the minor proteins present in egg white, this was subjected to an enrichment protocol, as explained in the Materials and Methods section, to increase the relative amount of minor proteins before the separation by 2DE. Fig. 1A shows the tentative identification of the proteins in the resultant 2D gel, following Guerin-Dubiard et al. [5]. The most abundant proteins, such as OVA, OM and LYS appeared as well defined spots corresponding to their different isoforms. However, OVT was poorly detected in the 2DE gel, probably as a result of the enrichment process, based on bead-coupled peptide libraries, which would reduce the most abundant proteins rather than equalizing the concentration of minor and major components [23].

Egg white proteins in the gel were electroblotted onto a nitrocellulose membrane and incubated with a pool of sera from egg-allergic subjects (Fig. 1B). The proteins that presented the highest binding to IgE were OVA, OVA-Y and LYS isoforms. OVA, a glycoprotein with a molecular mass of 45 kDa that exists in three levels of phosphorylation and constitutes the most abundant protein in egg white (54%), is

regarded as a major allergen [24]; while OVA-Y, with a molecular mass of about 53 kDa [25], presents 57% identity with OVA and bears OM-like carbohydrate chains [26]. The immunoreactivity of LYS, which is also regarded as an important egg allergen [24], against IgE was outstanding. Guérin-Dubiard et al. [5] reported the 2D separation of LYS spots corresponding to monomer and dimer forms of the protein. We also found a third spot, which also presented a high IgE binding, with the same apparent isolectric point but a molecular mass that could correspond to the trimer. Finally, OM, considered the immunodominant egg protein [24], also probably bound IgE, but OM isoforms, which appeared as diffuse bands with a higher apparent molecular mass than its real mass as a result of its high degree of glycosylation, were probably masked by OVA spots.

Two minor proteins tentatively identified as ovoinhibitor (OvoI) and clusterin following Guerin-Dubiard et al. [5], also reacted against the pool of human sera. OvoI accounts for 1.5% of total egg white protein and presents 22.5% of sequence identity with OM. Cross-reactivity between these proteins has been demonstrated using a rabbit anti-OM antibody [27], which suggests that antigenic determinants may be located in homologous regions. Indeed, several OM IgE epitopes [28] are found within regions that present sequence homology with OvoI kazal-like domains 1, 2 and 7, and one study has shown that OvoI strongly reacts with serum from hen egg allergic patients in immunodot assays [29]. Clusterin, previously detected in egg white, is a molecular chaperone, which is a protein that stabilises partially folded proteins to prevent their aggregation and precipitation [30]. Hitherto, its IgE reactivity has not been reported.

3.2 Residual immunoreactivity of egg white proteins following in vitro gastroduodenal digestion

The susceptibility of egg white proteins to in vitro gastroduodenal digestion and their residual immunoreactivity were investigated by 1D/2D-PAGE and Western blotting. Figure 2A shows that OVA and LYS were very stable to pepsin action during the gastric phase. Intact OVA and LYS remained after the duodenal phase, with the appearance, at this stage, of a number of resistant fragments within a wide range of molecular masses.

Western blotting with a polyclonal OVA-specific antibody confirmed the high resistance of this protein to gastrointestinal digestion and allowed the detection of its degradation products (Figure 2 B). We have previously shown that, in the absence of other egg-white proteins, OVA persists for 60 min of duodenal digestion even though the presence of bile salts at physiological concentrations considerably increase its hydrolysis [10]. A comparably higher content of intact OVA was found following duodenal hydrolysis of whole egg white (Figures 2 A and B) which could be attributed to the residual trypsin inhibitory activity of OM, albeit reduced by pepsin digestion [18, 31]. Purified LYS is also very resistant to pepsin digestion but it precipitates under simulated duodenal conditions, an effect that is ascribed to the bile salts [12]. Western blotting with a polyclonal LYS-specific antibody revealed that only a small amount of the intact protein was present after the duodenal phase (not shown). This, together with the virtual absence of detectable breakdown products, suggested that LYS precipitation also occurred during the in vitro duodenal digestion of egg white.

Western blotting evidenced polyclonal OM-specific antibody binding to intact OM in the gastric and duodenal digests (Fig. 2 C), indicating that this generally pepsin-labile protein could be protected from the enzymatic action in the egg white matrix. In fact, OM disappearance was confirmed after 10 min of gastric digestion when the protein was tested alone (results not shown), which was in agreement with previous results [11, 31]. It should be noticed that the band of around 14 kDa, observed in the gel and attributed to LYS (Fig. 2A, lane 3), cross reacted with the OM-specific antibody, probably due to the difficulty inherent in obtaining pure OM preparations for immunization [32].

IgE-binding capacity of the egg white gastroduodenal digest is shown in the immunoblot of Fig. 2D. The remaining OVA and the fragments named a-f strongly reacted against serum IgE, pointing out the important residual immunoreactivity of egg white allergens after digestion. These peptides were cut off from the gel and subjected to tryptic digestion and MS analyses. As shown in Table 1, almost all IgE-reactive bands were found to derive from OVA. Intact LYS and a LYS-derived peptide produced after digestion were also identified as responsible for the IgE-binding. To our surprise, no OM-derived peptides were identified in the IgE-reactive bands. Other authors [33] have also failed to identify OM by MALDI-TOF mass spectrometry, ascribing this difficulty to the protein stability against denaturation and trypsinization. However, it cannot be discarded that bands a, c, d and e would also contain OM-derived fragments, since they reacted against the OM-specific antibody (Fig. 2C, lane 3).

The IgE-reactive peptides present in the egg white gastroduodenal digest were also examined by 2D electrophoresis (Fig. 3A), followed by Western blotting (Fig. 3B). As a means to avoid predominant IgE-binding to OVA, the nitrocellulose membrane

was cut and only the transferred proteins below 30 kDa were probed against a pool of sera from egg-allergic patients. Several spots (highlighted in Fig. 3B), of around 25 kDa and within an apparent isoelectric point range of 5-6, produced the strongest binding signals. These spots were cut off from the 2DE gel and trypsinized for MS identification (Table 1, spots H-N). The most reactive fragments were found to derive from OVA in agreement with the fragments identified following SDS-PAGE (Fig. 2 D). Neither OvoI nor clusterin or IgE-reactive fragments deriving form these two proteins were detected in the immunoblot, what suggests their degradation and loss of immunoreactivity during the enzymatic digestion.

3.3 Residual immunoreactivity following *in vitro* digestion of egg white and yolk

In order to assess the effect of the egg yolk matrix on the susceptibility of egg allergens to digestion, we compared the *in vitro* hydrolysis of egg white in the absence and presence of egg yolk (Fig. 4 A and B). Digestion of egg yolk proteins is shown in Fig. 4B (lanes 7-9). Stacked proteins at the top of the gel probably corresponded to yolk granular proteins, such as the major complex lipovitelin-fosvitin [34] and bands of molecular weight >50 kDa to soluble fragments of the highly abundant LDL apoprotein ApoB, as deduced from their apparent molecular masses. In fact, Jolivet et al. [35] reported the existence of Apo B internal and C-terminal fragments of molecular mass 86, 64 and 55 kDa with a migration pattern similar to that shown in Fig. 4B lane 7. α-livetin (Gal d 5), a very abundant yolk plasma protein that is also considered an allergen [36], is probably the prominent band of, approximately, 70 kDa, indicated with an asterisk in Fig. 4B. Pepsin partially cleaved it during gastric digestion and it became completely hydrolyzed by duodenal enzymes, either in the absence or presence of egg

white (Fig. 4B, lanes 4-6), suggesting that α-livetin is a digestion-labile allergen in whole egg.

Regarding the hydrolysis of egg white proteins, OVA was very resistant to digestive enzymes, both in the absence or presence of egg yolk (Figure 4A, lanes 1-6, and Figure 4B), while OVT quickly disappeared after gastric digestion in both systems (Figure 4B). OM was hydrolyzed upon gastric digestion, but its complete disappearance could not be confirmed by SDS-PAGE or RP-HPLC. Of note is the peak corresponding to intact LYS after duodenal digestion that was just found by RP-HPLC in the presence of yolk (Figure 4B). Similarly, in Figure 4A, the band corresponding to LYS after duodenal digestion was more intense in the mixed egg white/yolk sample (arrows in lanes 3 and 6). This suggests that LYS precipitation due to bile salts could be prevented by yolk components. LYS interaction with bile salts is likely to be driven by electrostatic forces between the highly cationic protein and the anionic bile salt molecules. However, in the presence of the yolk, low density lipoproteins (LDL, accounting for 66% of total yolk dry matter) would presumably bind bile salts, leaving less surfactant molecules available to induce LYS aggregation. In fact, human LDL have been shown to possess 5-9 times more binding sites for bile salts per mg protein than the main carrier in blood albumin [37]. There is also a high concentration of PC in egg yolk (approximately 1.7 mmol) and the presence of PC partially avoids LYS precipitation induced by bile salts [12]. It can therefore be inferred that LYS in whole egg remains soluble under duodenal conditions and resists proteolysis to a large extent.

In general terms, while egg yolk did not exert a major influence on the digestion of egg white proteins, it could increase their susceptibility to hydrolysis in view of the differences observed in the digestion profiles: mainly, the higher intensity of the bands

corresponding to degradation products in SDS-PAGE (Fig 4A, lanes 2, 3 and 4, 5) or the higher number and concentration of peptides eluting between min 20 and 35 (Fig. 4B) in RP-HPLC. It has been reported that proteins like β-lactoglobulin or β-casein are able to adsorb at the interfaces of model oil-in-water emulsions and become more susceptible to enzymatic digestion [16]. However, lipids in the egg yolk are associated with lipoprotein assemblies and, therefore, they do not form interfaces where proteins could adsorb. Moreover, our results suggest that lipids potentially released from lipoproteins after digestion (mostly triglycerides and phospholipids) would not considerably affect the cleavage of egg white allergens by gastroduodenal enzymes.

Whether these differences could imply changes in immunoreactivity was addressed by comparing the IgE-binding of gastric and duodenal digests of egg white, egg yolk and the mixture of both of them by inhibition ELISA, using two different serum pools (3 subjects each) (Fig. 5). The digestion of egg yolk produced the least reactive hydrolysates, in accordance with the presence of dominant allergens in the egg white. However, no significant differences were found between the egg white digests and those obtained in the presence of egg yolk. Moreover, in both cases, gastric and duodenal digests had comparable IgE-binding capacities despite the higher extent of proteolysis of allergens, especially OVA (Fig. 4), in the latter, which could be attributed to the persistent fragments produced during duodenal digestion bearing IgE epitopes (Fig. 2D and 3B).

4. CONCLUDING REMARKS

Two minor proteins in egg white, tentatively identified as clusterin and ovoinhibitor, were found able to bind IgE from egg-allergic patients, although their

immunoreactivity was lost after simulated gastrointestinal digestion. OVA considerably resisted gastrointestinal digestion in egg white, giving rise to several IgE-reactive peptides. The presence of egg yolk slightly increased the susceptibility to hydrolysis of egg white proteins and reduced bile salt-induced precipitation of LYS in the duodenal medium what suggests that LYS, a highly immunoreactive protein, may remain soluble when whole egg is ingested resisting enzymatic proteolysis. However, the resultant immunoreactivity against IgE of egg white proteins after *in vitro* digestion was not significantly modified by the presence of yolk components.

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5. REFERENCES

[1] Rona, R.J., Keil, T., Summers, C., Gislason, D., et al. The prevalence of food allergy: a meta-analysis. *J. Allergy Clin. Immunol.* 2007, *120*, 638-646.

[2] Schade, R., and Chacana, P.A. In: Huopalahti, R., López-Fandiño, R., Anton, M. and Schade, R. (Eds.), Bioactive Egg Compounds-Livetin fractions (IgY) 2007, Springer, pp, 25-32.

[3] Amo, A., Rodríguez-Pérez, R., Blanco, J., Villota, J., et al. Gal d 6 Is the Second Allergen Characterized from Egg Yolk. *J. Agric. Food Chem*. 2010, *58*, 7453-7457.

[4] Li-Chan, E., and Nakai, S. Biochemical basis for the properties of egg white. *Crit. Rev. Poult. Biol*. 1989, *2*, 21-58.

[5] Guerin-Dubiard, C., Pasco, M., Molle, D., Desert, C., et al. Proteomic analysis of hen egg white. *J. Agric. Food Chem.* 2006, *54*, 3901-3910.

[6] Mann, K., and Mann, M. In-depth analysis of the chicken egg white proteome using an LTQ Orbitrap Velos. *Proteome Sci.* 2011, *9*, 7.

[7] Moreno, F.J. Gastrointestinal digestion of food allergens: effect on their allergenicity. Biomed. Pharmacother. 2007, 61, 50-60.

[8] Fu, T.J. Digestion stability as criterion for protein allergenicity assessment. Ann. New York Acad. Sci. 2002, 964, 99-110.

[9] Moreno, F.J., Mackie, A.R., Mills, E.N.C. Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. *J. Agric. Food Chem.* 2005, *53*, 9078-9082.

[10] Martos, G., Contreras, P., Molina, E., Lopez-Fandiño, R. Egg White Ovalbumin Digestion Mimicking Physiological Conditions. *J Agric. Food Chem*. 2010, *58*, 5640-5648.

[11] Jimenez-Saiz, R., Belloque, J., Molina E., Lopez-Fandino R. Human immunoglobulin E (IgE) binding to heated and glycated ovalbumin and ovomucoid before and after in vitro digestion. *J. Agric. Food Chem*. 2011, *59*, 10044-10051.

[12] Jimenez-Saiz, R., Martos, G., Carrillo, W., Lopez-Fandiño, R., Molina, E. Susceptibility of lysozyme to in-vitro digestion and immunoreactivity of its digests. *Food Chem*. 2011, *127*, 1719-1726.

[13] Nowak-Wegrzyn, A., Fiocchi, A. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. *Curr. Opin. Allergy Clin. Immunol.* 2009, *9*, 234-237.

[14] Teuber, S.S. Hypothesis: the protein body effect and other aspects of food matrix effects. *Ann. N. Y. Acad. Sci.* 2002, *964*, 111-116.

[15] Polovic, N., Blanusa, M., Gavrovic-Jankulovic, M., Atanoskovic-Markovic, M., Burazer, L., Jankov, R., Cirkovic Velickovic, T. A matrix effect in pectin-rich fruits hampers digestion of allergen by pepsin in vivo and in vitro. *Clin. Exp. Allery,* 2007, *37*, 764-771.

[16] Macierzanka, A., Sancho, A.I., Mills, E.N.C., Rigby, N.M., Mackie, A.R. Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin. *Soft Matter* 2009 *5*, 538-550.

[17] Maleki, S.J., Viquez, O., Jacks, T., Dodo, H., Champagne, E.T., Chung, S.-Y-., Landry, S.J. The major peanut allergen, Ara h2, functions as a trypsin inhibitor, and roasting enhances this function. *J. Allergy Clin. Immunol.* 2003, *112*, 190-195.

[18] Takagi, K., Teshima, R., Okunuki, H., Itoh, S., Kawasaki, N., Kawanishi, T., Hayakawa, T., Kohno, Y., Urisu, A., Sawada, J. Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. *Int. Arch. Allergy Immunol.* 2005, *136*, 23-32.

[19] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248-254.

[20] Mortz, E., Krogh, T.N., Vorum, H., Gorg, A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics* 2001, 1, 1359-1363.

[21] Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols* 2006, *1*, 2856-2860.

[22] Quirós, A., Chicón, R., Recio, I., López-Fandiño, R. The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chem.* 2007, *104*, 1734-1739.

[23] Keidel, E.M., Ribitsch, D., Lottspeich, F. Equalizer technology - Equal rights for disparate beads. *Proteomics* 2010, *10*, 2089-2098.

[24] Mine, Y., Yang, M. Recent advances in the understanding of egg allergens: basic, industrial, and clinical perspectives. *J. Agric. Food Chem.* 2008, *56*, 4874-4900.

[25] Nau, F., Pasco, M., Desert, C., Molle, D., et al. Identification and characterization of ovalbumin gene Y in hen egg white. *J. Agric. Food Chem.* 2005, *53*, 2158-2163.

[26] Hirose, J., Doi, Y., Kitabatake, N., Narita, H. Ovalbumin-related gene Y protein bears carbohydrate chains of the ovomucoid type. *Biosci. Biotech. Biochem*. 2006, *70*, 144-151.

[27] Matsuda, T., Watanabe, K., Nakamura, R. Ovomucoid and Ovoinhibitor Isolated from Chicken Egg-White Are Immunologically Cross-Reactive. *Biochem. Biophys. Res. Commun.* 1983, *110,* 75-81.

[28] Mine, Y., Zhang, J.W. Identification and fine mapping of IgG and IgE epitopes in ovomucoid. *Biochem. Biophys. Res. Commun.* 2002, *292*, 1070-1074.

[29] Peng, H.J. Studies of allergenicity of hen egg ovoinhibitor. *Clin. Immunol.* 2002, *103*, S115-S116.

[30]. Mann, K., Gautron, J., Nys, Y., McKee, M.D., et al. Disulfide-linked heterodimeric clusterin is a component of the chicken eggshell matrix and egg white. Matrix Biol. 2003, *22*, 397-407.

[31] Kovacs-Nolan, J., Zhang, J.W., Hayakawa, S., Mine, Y. Immunochemical and structural analysis of pepsin-digested egg white ovomucoid. *J. Agric. Food Chem.* 2000, *48,* 6261-6266.

[32] Pellegrini, A., Grob, K., Vonfellenberg, R. Identification of a Bactericidal Component Active against Gram-Positive and Gram-Negative Bacteria in Commercial Ovomucoid and Ovoinhibitor as Lysozyme. *Lett. Appl. Microbiol*. 1990, *10*, 201-204.

[33] Lee, J.Y., Kim, C.J. Determination of Allergenic Egg Proteins in Food by Protein-, Mass Spectrometry-, and DNA-Based Methods. J. AOAC Int. 2010, *93*, 462-477.

[34] Wallace, R.A. The Vertebrate Ovary, ed. R.E. Jones. 1978, New York: Plenum.p 469-502.

[35] Jolivet, P., Boulard, C., Beaumal, V., Chardot, T., Anton, M. Protein components of low-density lipoproteins purified from hen egg yolk. *J. Agric. Food Chem.* 2006, *54,* 4424-4429.

[36] Quirce, S., Maranon, F., Umpierrez, A., de las Heras, M., et al. Identification of chicken serum albumin as a thermolabile egg allergen (Gal d 5) responsible for the bird-egg syndrome. *J. Allergy Clin. Immunol*. 2000, *105*, S136-S137.

[37] Ceryak, S., Bouscarel, B., Fromm, H. Comparative Binding of Bile-Acids to Serum-Lipoproteins and Albumin. *J. Lipid Res*. 1993, *34,* 1661-1674.

Table 1. *Protein identification of immunoreactive bands and spots by peptide mass fingerprinting. All scores were significant according to Mascot Search Engine ($p<0.05$).*

Bands/spots	Identification	Coverage	Peptides matched	Score
a	Ovalbumin	24%	6	63
b	Ovalbumin	34%	11	99
c	Ovalbumin	39%	13	85
d	Ovalbumin	32%	10	72
e	Lysozyme C	63%	12	122
f	Ovalbumin	20%	7	212
	Lysozyme C	13%	2	81
H	Ovalbumin	33%	16	631
I	Ovalbumin	35%	12	85
J	Ovalbumin	45%	17	127
K	Ovalbumin	35%	14	85
L	Ovalbumin	29%	11	253
M	Ovalbumin	35%	12	83
N	Chymotrypsin bovine	15%	4	143

FIGURE LEGENDS

Figure 1. *A) 2D PAGE of egg white proteins. B) Immunoblot of egg white proteins using a serum pool of egg allergic patients (Mean specific IgE level to egg white: 13.2 kU/L).*

Figure 2. *SDS-PAGE (A) and Western blotting (B, C and D) of egg white proteins: egg white (1), gastric digest (2) and gastroduodenal digest (3). MW: molecular weight marker. Membranes were probed against ovalbumin antibody (B, anti-OVA), ovomucoid antibody (C, anti-OM) antibodies, and a serum pool (D, IgE) of egg-allergic patients (mean specific IgE level to egg white: 70.5 kU/L). IgE-binding fragments are indicated by letters a-f.*

Figure 3. *A) 2DE of egg white gastroduodenal digest. Spots H-N were analyzed by mass spectrometry. B) Immunoblot of fragments lower than 30 kDa from egg white gastroduodenal digest using a serum pool of egg-allergic patients (mean specific IgE level to egg white: 70.5 kU/L). MW: Molecular weight marker. IgE-binding spots are shown in circles.*

Figure 4. *A) SDS-PAGE of undigested (1, 4, 7), gastric digests (2, 5, 8) and gastroduodenal digests (3, 6, 9) of egg white (EW, lanes 1-3), the mixture of egg white and yolk (EW+Y, lanes 4-6) and egg yolk (Y, lanes 7-9). MW: Molecular weight marker. * indicates α-livetin. B) HPLC chromatograms of gastric and duodenal digests of the mixture of egg white (solid line) and the mixture of egg white and yolk (dotted line).*

Figure 5. *Inhibition ELISA of gastric (G60) and gastroduodenal digests (D30) of egg white (EW), egg yolk (Y), and the mixture of both (EW+Y). The concentration that binds 50% (IC50) of IgE from two different serum pools is represented. Mean specific IgE levels to egg white were 15.1 kU/L for pool 1 and 13.29 kU/L for pool 2 and IgE levels for egg yolk < 5kU/L). Error bars indicate the standard error of the mean.*

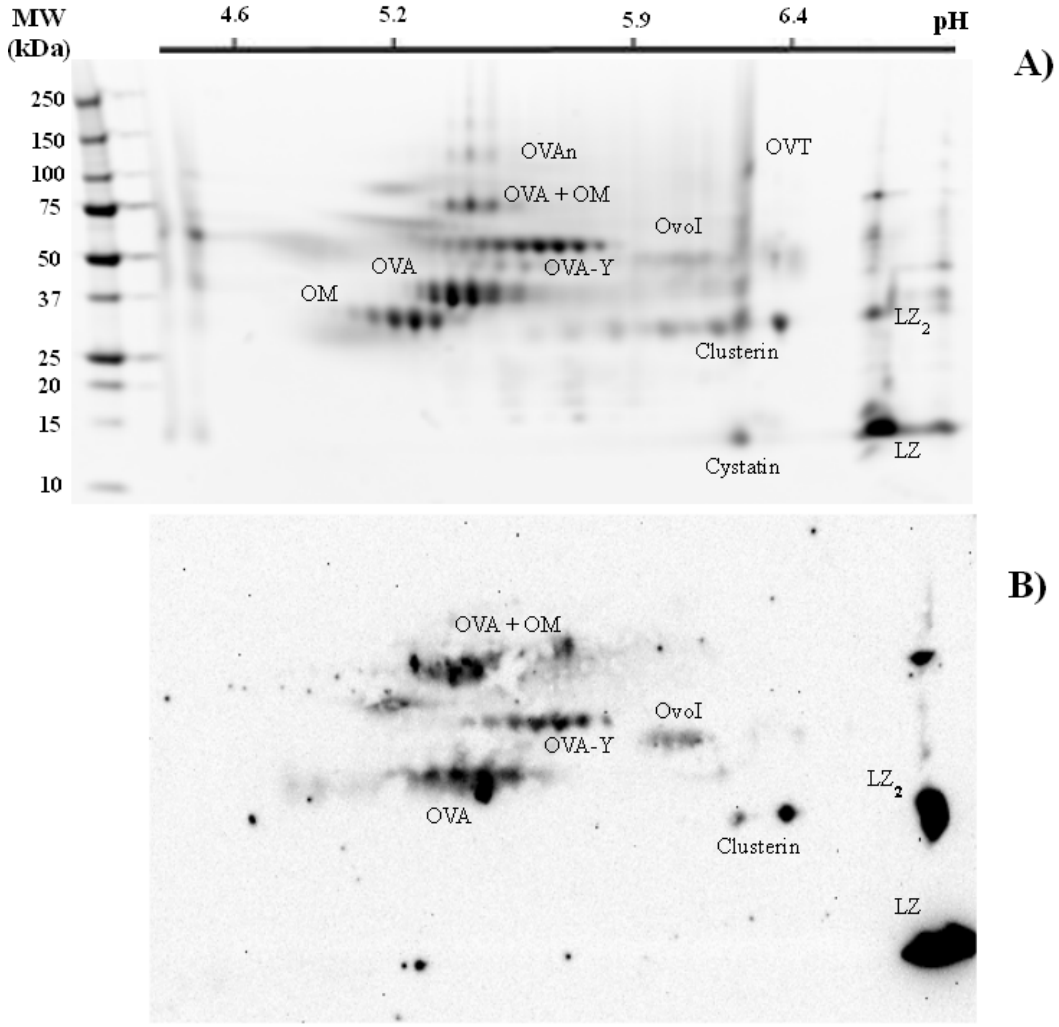

MW
(kDa)
4.6
5.2
5.9
6.4
pH
A)
250
150
100
75
50
37
25
20
15
10
OVAn
OVT
OVA + OM
OvoI
OVA
OVA-Y
OM
LZ2
Clusterin
LZ
Cystatin
B)
OVA + OM
OvoI
OVA-Y
LZ2
OVA
Clusterin
LZ

Figure 1

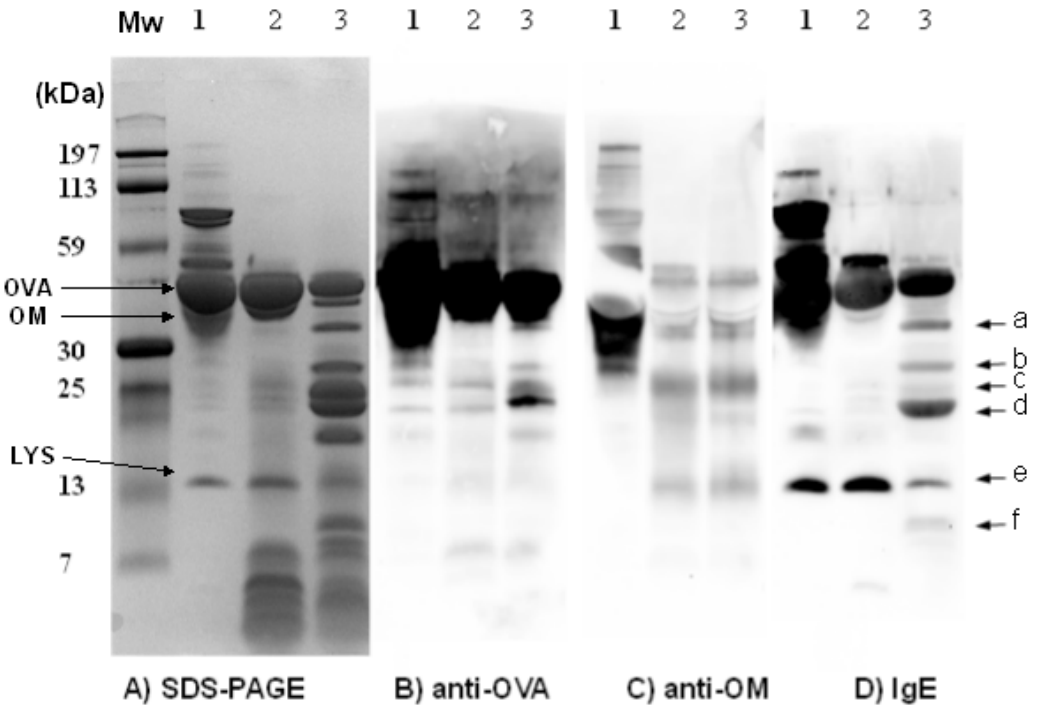

Mw
1
2
3
1
2
3
1
2
3
1
2
3
(kDa)
197
113
59
OVA
OM
30
25
LYS
13
7
a
b
c
d
e
f
A) SDS-PAGE
B) anti-OVA
C) anti-OM
D) IgE

Figure 2

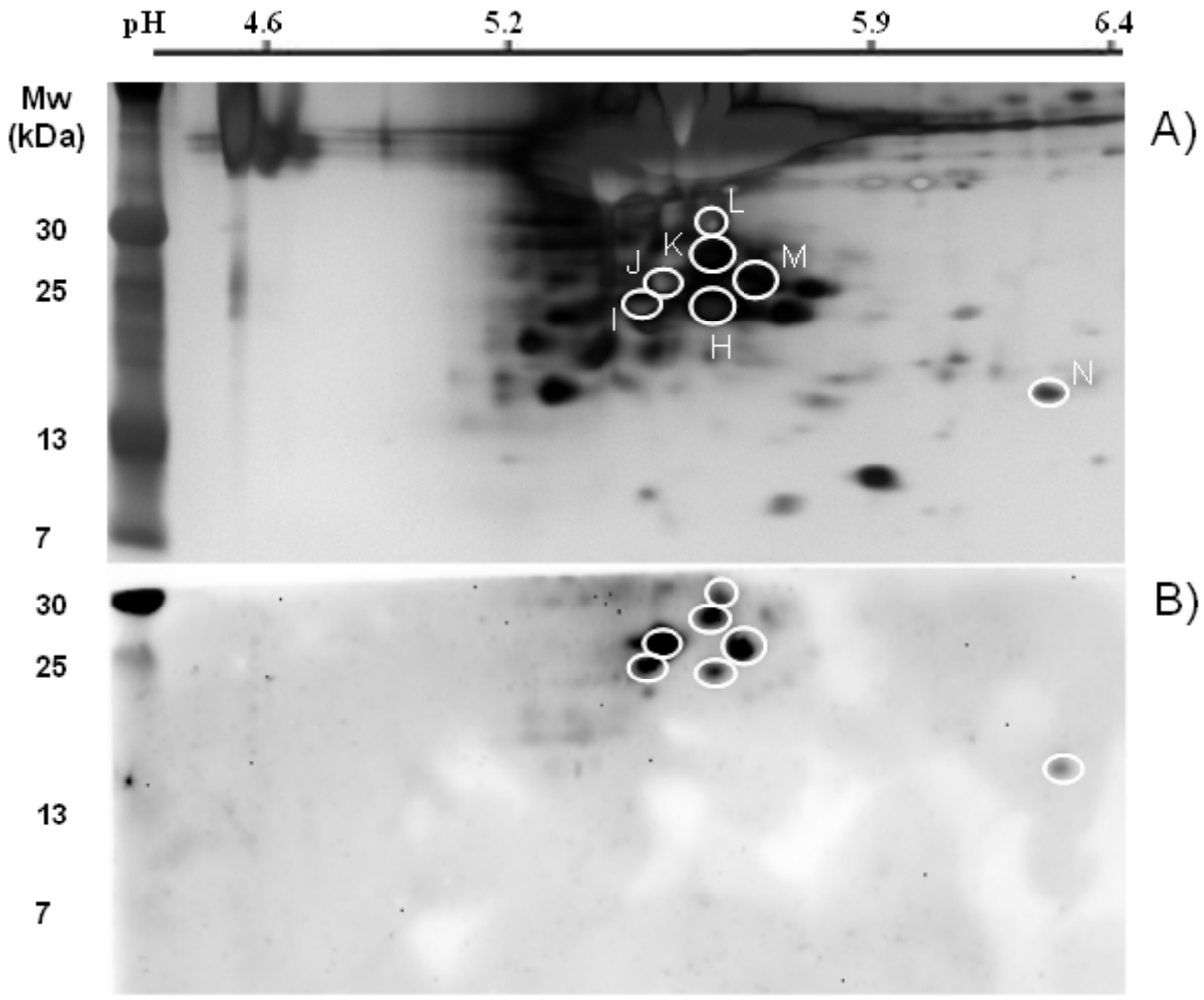


Figure 3

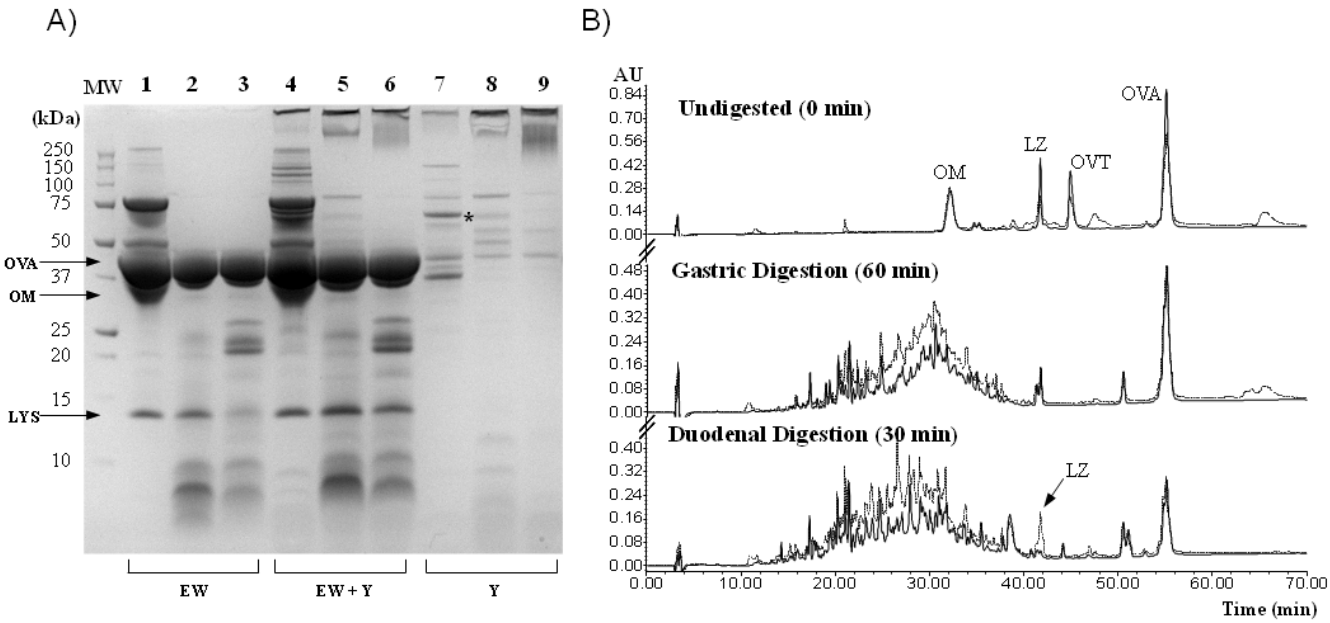

A)
B)
MW
1
2
3
4
5
6
7
8
9
(kDa)
250
150
100
75
50
37
25
20
15
10
OVA
OM
LYS
*
EW
EW+Y
Y
AU
Undigested (0 min)
OM
LZ
OVT
OVA
Gastric Digestion (60 min)
Duodenal Digestion (30 min)
LZ
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Time (min)

Figure 4

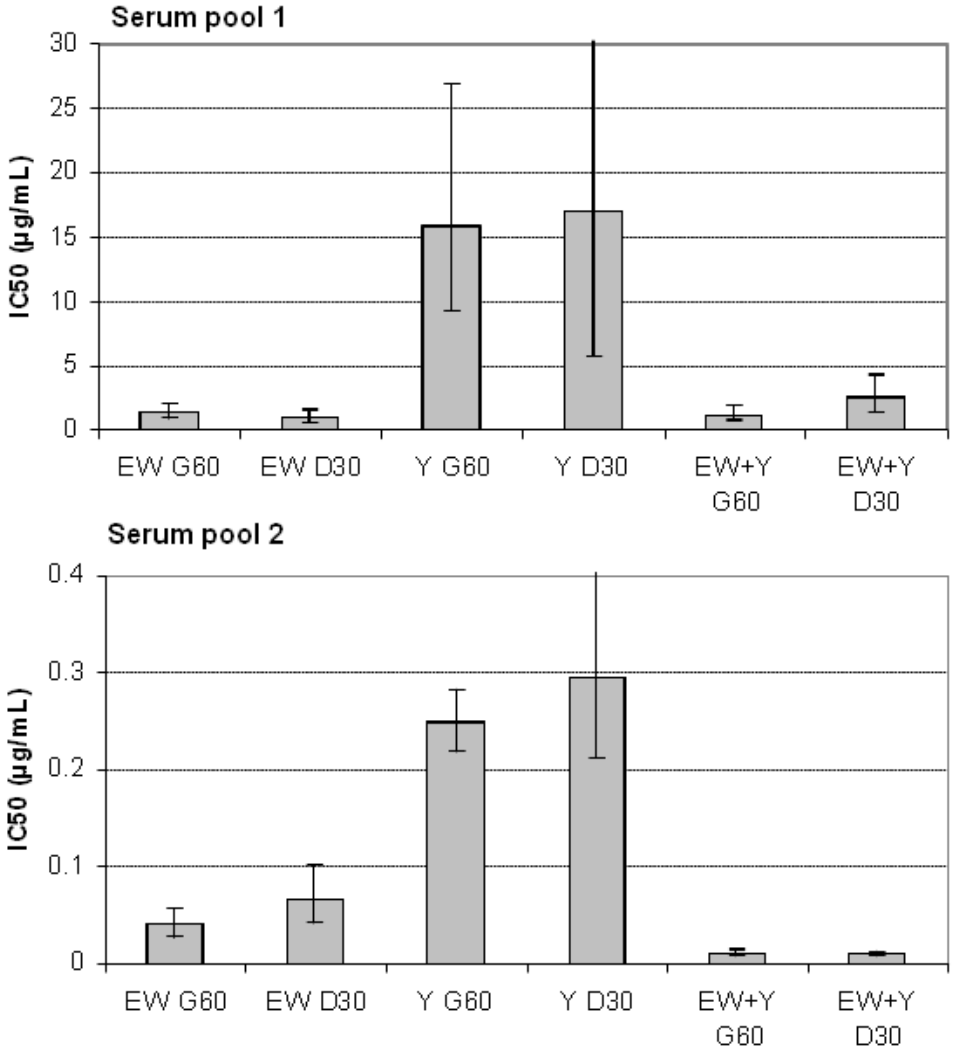
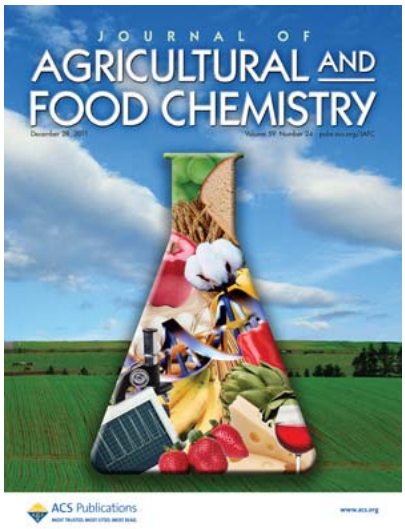

Serum pool 1
IC50 (µg/mL)
30
25
20
15
10
5
0
EW G60
EW D30
Y G60
Y D30
EW+Y G60
EW+Y D30
Serum pool 2
IC50 (µg/mL)
0.4
0.3
0.2
0.1
0
EW G60
EW D30
Y G60
Y D30
EW+Y G60
EW+Y D30

Figure 5

IV



J. Agric. Food Chem. (enviado)

Identification of an IgE reactive peptide in hen egg riboflavin binding protein subjected to simulated gastrointestinal digestion

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Identification of an IgE reactive peptide in hen egg riboflavin binding protein subjected to simulated gastrointestinal digestion.

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18 ABSTRACT

19

20 Riboflavin binding protein (RfBP) is a minor protein in hen egg, whose potential
21 involvement in egg allergy has seldom been studied. The aim of this work was to
22 investigate the IgE binding capacity of RfBP before and after a simulated
23 gastrointestinal digestion. The protein was cleaved mainly in the gastric phase of
24 digestion. After gastroduodenal digestion, the protein was fragmented but linked
25 through disulfide bonds. The intact protein and its digests were subjected to inhibition
26 ELISA with sera from patients allergic to egg. The results showed considerable IgE-
27 binding for intact RfBP, while the digests showed reduced but still relevant IgE-
28 binding, which varied with the serum used. The digests were then subjected to
29 immunoblot with allergic patients' sera, and the IgE-reactive peptides were further
30 analysed by MALDI-TOF/TOF mass spectrometry for identification. The results
31 showed that peptide 41-84 from RfBP digests was able to bind serum IgE from patients
32 allergic to egg.

33

34

35

36 *Keywords:* riboflavin binding protein, riboflavin carrier protein, RCP, ovoflavoprotein,
37 digestion, proteolysis, egg white, allergen, epitope, peptide

38

39

40 INTRODUCTION

41

42 Allergy to egg is very common among food allergies, particularly in children,
43 affecting 1.5-3.2 % of this population[1]. There are well known egg allergens, such as
44 ovalbumin (*Gal d 2*), ovomucoid (*Gal d 1*), ovotransferrin (*Gal d 3*) and lysozyme (*Gal*
45 *d 4*) in the egg white, or α-livetin (*Gal d 5*) in the egg yolk and, recently, a fragment of
46 the vitellogenin-1 precursor has been identified as another yolk allergen, (*Gal d 6*)[2]. Due
47 to the diversity of the allergic response, regarding specificity and degree of sensitivity to
48 egg allergens, and the presence of many minor egg proteins that still remain poorly
49 characterized[3], other egg proteins or their fragments may be involved in the allergic
50 response, either as sensitizing allergens or through cross-reactions.

51 Riboflavin binding protein (RfBP), also named riboflavin carrier protein,
52 ovoflavoprotein or flavoprotein, is known for its involvement in reproductive biology[4].
53 In hen egg, RfBP is present in both white and yolk in amounts of 0.8 and 0.3 % (w/w
54 protein) respectively[5]. It is a small acidic and heat-stable protein, whose molecular
55 properties have been reviewed elsewhere[6-8]. It is 219 amino acids long, its sequence
56 exhibits an unusual pyroglutamic residue at the N-terminal end, and it contains linked
57 carbohydrates that account for ~14% of its molecular weight (30-35 kDa), phosphate
58 moieties that contribute to its pI of ~4.0, and nine disulfide bonds, which make it very
59 stable to thermal denaturation. It has been regarded as a minor allergen[9], but there is not
60 much literature about its allergenic potential. Some authors have found cross reactivity
61 between bovine caseins and RfBP[10] and previous work in our laboratory, using different
62 sera of patients allergic to egg (unpublished), revealed that RfBP exhibited high IgE
63 binding by indirect ELISA. The allergenic potential of RfBP should be taken into
64 consideration, not only because egg products are widely distributed in foods, but also

65 because recent studies have proposed the use of RfBP to inhibit bitterness[11], which
66 could lead to new food applications where it could be found as a hidden allergen.
67 It is generally accepted that digestion of a protein along the gastrointestinal (GI)
68 system significantly alters its allergenicity and, therefore, it is convenient to assess the
69 potential allergenicity of a protein after digestion. *In vitro* assays for digestibility have
70 evolved in the last decade by introducing variables that intend to approach the digestion
71 model to the physiological process[12]. *In vitro* models that include subsequent gastric and
72 duodenal steps, a better fit of the pH and the E:S ratio, and incorporation of other
73 important components, such as phospholipids or bile salts, have been used to study egg
74 allergens[13,14].
75 In order to investigate the potential involvement of RfBP in egg allergy, the aim
76 of this study was to evaluate the IgE binding capacity of intact RfBP and RfBP
77 digestion products obtained from an *in vitro* model that mimics physiological
78 conditions.
79
80 **MATERIALS AND METHODS**
81
82 ***In vitro* gastroduodenal digestion.** In vitro digestions were carried out in
83 duplicate. Riboflavin binding protein from chicken egg white (Apo form, Sigma, MO,
84 USA) was digested according to the method reported by Moreno and col.[12], and
85 modified by Martos and col.[13], using the enzyme pepsin for the gastric phase and
86 trypsin and α-chymotrypsin for the subsequent duodenal phase. Aliquots were taken at 0
87 and 60 min of gastric digestion and at 30 and 60 min of the duodenal digestion. Aliquots
88 of digests were also taken after 24 h of gastric digestion. Eventually, all samples were

89 diluted in milli-Q water to a final protein concentration of 1.24 mg/mL and kept at -20
90 ºC until further use.
91
92 **RP-HPLC.** Aliquots taken during gastroduodenal digestion were filtered
93 through 0.2 μm nitrocellulose filters and analyzed by HPLC[13], using a Hi-Pore RP-318
94 (250 x 4.6 mm) column (Bio-Rad) in a Waters 600 HPLC (Waters Corporation,
95 Milford, MA) equipped with a 717 plus autosampler and a 2487 dual wavelength
96 absorbance detector. The mobile phases used were 0.37% (v/v) trifluoroacetic acid
97 (TFA) in milli-Q water as solvent A and, 0.27% (v/v) TFA in acetonitrile as solvent B.
98 A linear gradient of 0-60% B was applied over 60 min. After 35 min at 60% B, 100% B
99 was reached in 1 min, maintained during 10 min and finally decreased to 0% in 1 min.
100 The flow rate of mobile phase was maintained at 1 ml/ min and peptides were detected
101 by monitoring absorbance at 220 nm. Data were processed by using Empower 2
102 Software (Waters Corporation).
103
104 **SDS-PAGE.** Proteins and peptides were separated according to their molecular
105 weight on Precast Criterion XT 4-12% Bis-Tris gels using the Criterion cell (Bio-Rad,
106 Richmond, CA, USA). Samples were diluted 1:3 (v/v) in a buffer containing 62.5 mM
107 Tris-HCl pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol and
108 0.025 % (w/v) bromophenol blue. The same buffer without β-mercaptoethanol was used
109 for separations under non-reducing conditions. Samples were then heated at 95 ºC for 4
110 min and 40 μL were loaded on a 4-12 % Bis-Tris polyacrylamide gel (CriteriumTMXT,
111 Bio-Rad). Electrophoretic separations were carried out at 100 V in a Criterion cell using
112 XT-MES as running buffer (Bio-Rad). Gels were stained with Coomassie blue G-250.
113

Collection of allergic patient's sera. Serum samples from children with proven allergy to egg white were collected with written consent at the Hospital Gregorio Marañón (Madrid, Spain). All patients showed IgE specific towards egg white, as estimated by the Immuno-CAP method (Pharmacia Diagnostic, Uppsala, Sweden) and exhibited clinical allergic symptoms. All procedures regarding serum collection and patient's confidential data treatment were done according to current laws and approved by the ethics committee of the hospital.

Western Blot. Samples of interest were subjected to electrophoresis as described above. Afterwards, the gel was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20 % methanol, pH 9.2) for 20 min, and blotted on a nitrocellulose membrane using a Trans-Blot semidry system (Bio-Rad) for 30 min at 18 V. Then, blocking was carried out using 1 % (w/v) bovine serum albumin (BSA) in Tris buffered saline containing 0.05 % (v/v) Tween 20 (TBS-T). After washing for 5 min with TBS-T, the membrane was dipped into 5 mL of patient's serum (a pool of sera with an averaged IgE titre of 13.2 KU/L for egg white, further diluted 1:30 in blocking buffer) and kept for 40 h at 4 ºC. After four washes, it was incubated for 6 h at 4 ºC with biotin-conjugated anti-human-IgE antibody (Southern Biotech, Birmingham, AL, USA) diluted 1:500 in blocking buffer. Again, the membrane was washed four times. Then, HRP-Streptavidin (DakoCytomation, Glostrup, Denmark) diluted 1:3000 in blocking buffer, was added and incubated for 1 h at room temperature (RT). Finally, the membrane was washed and revealed with the chemiluminescent substrate ECL Prime WB reagent (Amersham Biosciences, Inc). Image acquisition was done in a VersaDoc Imaging System (Bio-Rad), using an exposure time of 10 min.

IgE binding by inhibition ELISA. Inhibition ELISA was conducted following a previously reported procedure[15]. Polystyrene microtiter plates (Corning, Cambridge, MA, USA) were coated with RfBP (10 μg/mL) in phosphate buffered saline solution (PBS) and incubated overnight at 4 ºC. Plates were blocked with PBS containing 2.5% Tween 20 and incubated overnight at 4 ºC. On the other hand, 11 serial dilutions (starting at 2 mg/mL) of the digested and undigested protein were prepared in duplicate. Each dilution was mixed with an equal volume of patient's serum (diluted 1/200 in PBS containing 0.05% Tween 20), incubated for 2 h at RT and then added to the precoated wells. Plates were then incubated for 2 h at RT. Then, rabbit anti-human IgE antibody (DakoCytomation, diluted 1:1000) was added and incubated for 1 h at RT. This was followed by the addition of HRP-conjugated swine anti-rabbit IgG antibody (DakoCytomation, diluted 1:2000), incubated for 1 h at RT, and the use of a tyramide-biotin-streptavidin-HRP amplification system (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Walthman, MA, USA) following the instructions of the manufacturer. 3,3',5,5'-tetramethyl-benzidine (TMB, Sigma-Aldrich) was used as substrate for HRP, and the reaction was stopped with sulphuric acid. Absorbance was measured at 450 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

ELISA determinations were carried out with 3 different pools, each containing sera from three different allergic individuals with average IgE levels against egg white of 39.76, 43.09 and 39.5 kU/L. Antigen without antibody was used as maximum inhibition and antibody without antigen was used as minimum inhibition. A nonlinear adjustment of the data obtained was applied for each pool and sample. The adjustment model was a sigmoidal curve of inhibition dose-response with variable slope, from which the IC_{50} was obtained with the program GraphPad Prism® package (GraphPad

164 Software Inc., San Diego, CA, USA). The IgE binding capacity was expressed as
165 $1/IC_{50}$.
166
167 **Mass spectrometry analysis of peptide bands.** Assignment of peptides to a
168 specific protein was done by tryptic digestion and mass fingerprinting. The
169 identification of the exact sequence of particular peptides was done by mass
170 fingerprinting and MS/MS analysis of tryptic digests, in combination with intact
171 molecular weight determination.
172 Bands of interest were manually excised from micro preparative gels using
173 biopsy punches. Peptides selected for analysis were in-gel reduced, alkylated and
174 digested with trypsin[16]. After digestion, the supernatant was collected and spotted onto a
175 MALDI target plate with α-CHCA matrix (Sigma) for protein identification by peptide
176 mass fingerprint. SwissProt DB v. 57.15 with taxonomy restriction to Metazoa
177 (Animals) was searched using MASCOT 2.2 (matrixscience.com) through the Global
178 Protein Server v3.6 from ABSciex. Search parameters were carbamidomethyl cystein as
179 fixed modification and oxidized methionine as variable modification; peptide mass
180 tolerance 50 ppm and allowance for 1 missed trypsin cleavage site. Probability scores
181 were greater than the score fixed by MASCOT as significant with P-value<0.05.
182 Peptide sequences were confirmed by MS/MS analysis. Search parameters were
183 the same as described above. Mass tolerance for MS/MS fragments was set at 0.3 Da
184 and error tolerance for combined search (Peptide mass fingerprint plus MS/MS spectra)
185 was –80 ppm. Analyses were performed in a 4700 Proteomics MALDI-TOF/TOF
186 Analyzer (Perseptives Biosystems, Framingham, MA).
187 For intact molecular weight determination, proteins were electroblotted as
188 described above onto a nitrocellulose membrane and detected by Ponceau S staining

189 (Sigma). The bands of interest were excised and directly analyzed after dissolution of
190 the nitrocellulose in MALDI matrix solution prepared as a saturated solution of α-
191 CHCA in acetonitrile:methanol (70:30) containing 1 % TFA[17]. Samples were spotted on
192 a Bruker Anchorchip target and spectra were acquired on linear mode using a Bruker
193 Autoflex SpeedTM spectrometer (Bruker Daltonics Gmbh, Bremen, Germany).
194
195 **RESULTS AND DISCUSSION**
196
197 **Digestibility of RBP.** RP-HPLC and SDS-PAGE analyses of the simulated GI
198 digestion of RfBP are shown in Fig. 1 and 2 respectively. RfBP was mostly cleaved by
199 pepsin throughout gastric digestion, giving rise to peptides that eluted at lower retention
200 times in the chromatographic separation than the intact protein. These hydrophilic
201 peptides were more abundant as the digestion evolved through the following duodenal
202 step (Fig. 1). SDS-PAGE under non-reducing conditions showed the persistent presence
203 of the intact protein after all steps of the simulated digestion (Fig. 2, lanes 7-11).
204 However, when the digests were analyzed under reducing conditions (Fig. 2, lanes 1-5),
205 the main protein band completely disappeared after the gastric digestion step, giving
206 rise to new bands, corresponding to digestion products with lower molecular weight
207 (Fig. 2, lane 3). As RfBP contains 9 disulfide bridges within the molecule, these results
208 suggest that the full-length protein was cleaved during the gastric digestion step,
209 although its fragments remained linked together through disulfide bonds. In addition, in
210 the time frame employed, the whole simulated physiological digestion led to fragments
211 of considerable size (up to 15 kDa). After 24 h of gastric digestion the protein and its
212 fragments completely disappeared from the SDS-PAGE gels (Fig. 2, lanes 6 and 12).
213

214 **Immunoreactivity of RfBP digests.** In order to assess the human IgE
215 recognition of RfBP before and after *in vitro* gastrointestinal digestion, inhibition
216 ELISA assays were performed on the intact protein and on its digests, using three
217 different serum pools from egg-allergic patients, specific to RfBP (Fig. 3). Gastric
218 digests showed reduced IgE binding capacity, when compared to the undigested protein.
219 Duodenal digests also presented reduced immunoreactivity, albeit comparable to the
220 gastric ones. However, in general, a relevant residual IgE binding was found in the
221 digests, although it greatly varied depending on the serum pool used. Serum pool 1,
222 presenting the highest reactivity to the intact protein, exhibited also the highest
223 reactivity to the digests. Similarly, serum pool 2 showed a high residual reactivity to the
224 digests, while serum pool 3 did not react to any of the digests despite presenting RfBP-
225 specific IgE binding comparable to pool 2. Therefore, serum pools 1 and 2 from egg-
226 allergic patients were able to recognize RfBP even after 60 min of simulated duodenal
227 digestion.
228
229 **Detection and identification of IgE-reactive RfBP fragments.** In order to
230 know the fragments of digested RfBP responsible for the binding to serum IgE, an
231 immunoblot was performed using a pool of sera (Fig. 4). Prior to digestion, RfBP
232 showed a strong binding to IgE (Fig. 4B, lane 1). After digestion, four bands with
233 apparent molecular masses of 4, 5, 36 and 40 kDa, respectively (labelled as R1, R2, R3,
234 R/O in Fig. 4B), still preserved IgE binding epitopes, as evidenced in the immunoblot
235 (Fig. 4B, lanes 2 and 3). It was only after 24 h of incubation with pepsin that all IgE
236 binding sites were destroyed (Fig. 4B, lane 4).
237 As shown in the immunoblot, two bands with MW higher than RfBP were IgE-
238 reactive (labelled R3 and R/O in Fig. 4B), which could be due to the presence of other

239 allergens in the RfBP samples. By peptide mass fingerprinting, we found fragment R/O
240 to contain both RfBP and ovalbumin, while the other three bands (R1, R2 and R3) were
241 identified as RfBP. Therefore, even though we cannot preclude that the presence of
242 ovalbumin contributed to the IgE-binding, the fragments R1 and R2, recognized by
243 serum IgE of allergic patients, were unequivocally assigned to different fragments of
244 digested RfBP.

245 We next proceeded to investigate the sequence of fragments R1 and R2. These
246 peptides were transferred from the gel into a nitrocellulose membrane for intact mass
247 determination, giving approximate masses of 3.9 and 5.1 kDa, respectively. Moreover,
248 tryptic peptides produced after in-gel digestion of the two fragments are shown in Fig.
249 5. R2 was unequivocally identified as fragment 41-84, while R1 contained residues 63-
250 84, but it was not possible to find all peptides covering the whole sequence. However,
251 according to the estimated molecular weight of 3.9 kDa, it is very likely that this band
252 corresponds to residues 52/53-84. Therefore, this region of the protein could contain
253 one or multiple IgE binding epitopes that would partially resist gastrointestinal digestion
254 being large enough to potentially crosslink IgE on mast cells.

255 The carbohydrate moieties of RfBP and the dominant egg allergen ovomucoid
256 show high similarity[18], and it has been suggested that they may be involved in the cross-
257 reactivity of the two proteins with a monoclonal antibody[19]. However, other authors
258 have discarded the presence of epitopes on ovomucoid carbohydrates[20,21]. In RfBP, the
259 N-linked carbohydrates are found on Asn 36 and 147[22]. These positions are not included
260 in the immunoreactive peptide 41-84. Therefore, IgE binds the peptide chain, which
261 suggests that potential IgE cross-reactivity of RfBP and ovomucoid would not be likely
262 related to their carbohydrate moieties.

263 Even though extensive research has been performed in the last years with regard
264 to egg allergens, there are still many questions to be answered. Most efforts have
265 focused on the main allergens, ovalbumin and ovomucoid, but the role of other proteins
266 present in egg at lower concentrations has not been taken into consideration. In this
267 work, we assessed the capability of intact RfBP to bind IgE from egg allergic patients,
268 finding a positive response, which points at RfBP as a cross-reactive allergen. On the
269 other hand, we showed that the intact protein was cleaved by pepsin in the simulated
270 gastric digestion, but its fragments remained linked through disulfide bridges. This
271 could maintain together epitopes far apart in the sequence that could bind various IgE
272 molecules. Furthermore, it was found that after the simulated duodenal digestion, an
273 RfBP fragment that corresponded to residues 41-84, long enough to promote IgE cross-
274 linking, was reactive against patient's serum IgE. Subramanian and Adiga[23] have
275 identified by PEPSCAN analysis some antigenic B-cell epitopes for chicken RfBP
276 included in this sequence, particularly 42-49 and 68-83. In addition, the fragment 64-83
277 contains a bimodal T-cell epitope[24]. This suggests that this region of the protein may be
278 immunologically relevant at physiological conditions.
279 In conclusion, RfBP is able to bind IgE from egg allergic patients, and, even
280 though it is degraded in a physiological simulated digestion, particularly during the
281 gastric phase, at least one fragment that resists digestion, i.e. 41-84, contains epitopes
282 recognizable by allergic patient's IgE, that could contribute to the allergic response
283 towards hen egg.
284
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297 **LITERATURE CITED**

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299 1. Savage, J. H.; Matsui, E. C.; Skripak, J. M.; Wood, R. A. The natural history of
300 egg allergy. *J. Allergy Clin. Immun.* **2007**, *120*, 1413-1417.
301 2. Amo, A.; Rodriguez-Perez, R.; Blanco, J.; Villota, J.; Juste, S.; Moneo, I.;
302 Caballero, M. L. *Gal d 6* is the Second Allergen Characterized from Egg Yolk. *J. Agric.*
303 *Food Chem.* **2010**, *58*, 7453–7457.
304 3. Guérin-Dubiard, C.; Pasco, M.; Mollé, D.; Désert, C.; Croguennec, T.; Nau, F.
305 Proteomic analysis of hen egg white. *J. Agric. Food Chem.* **2006**, *54*, 3901-3910.
306 4. Adiga, P. R. Riboflavin carrier protein in reproduction. In *Vitamin Receptors*;
307 Dakshinamurti, K., Ed.; Cambridge University Press: Cambridge, UK, New York, NY,
308 Melbourne, Australia, 1994; pp. 137-176.
309 5. White, H. B. III; Armstrong, J.; Whitehead, C. C. Riboflavin-binding protein.
310 *Biochem. J.* **1986**, *238*, 671-675.
311 6. White, H. B. III; Merrill, A. H. Jr. Riboflavin Binding Proteins. *Ann. Rev. Nutr.*
312 **1988**, *8*, 279-299.
313 7. Monaco, H. L. Crystal structure of chicken riboflavin protein. *EMBO J.* **1997**,
314 *16*, 1475-1483.
315 8. Croguennec, T.; Guérin-Dubiard, C.; Nau, F. Riboflavin Binding Protein
316 (Flavoprotein). In *Bioactive Egg Compounds*; Huopalahti, R., López Fandiño, R.,
317 Anton, M., Schade, R. (Eds.), Springer-Verlag: Berlin, Germany, Heidelberg, Germany,
318 2007; pp. 69-74.
319 9. Lehrer, S. B.; Reese G. Applications of molecular biology in food allergy.
320 Allergens of animal origin. *Alergologia e Inmunologia Clinica* **2001**, *16 (S2)*, 20-25.

10. Peltre, G.; Bernard, H.; Grassi, J.; David, B.; Wal, J. M. Cross-Reaction between calcium-sensitive bovine caseins and riboflavin binding-protein in allergy to cow's milk-proteins. *J. Allergy Clin. Immun.* **1994**, *93*, 210-210.

11. Maehashi, K.; Matano, M.; Nonaka, M.; Udaka, S.; Yamamoto, Y. Riboflavin-binding protein is a novel bitter inhibitor. *Chem. Senses* **2008**, *33*, 57-63.

12. Moreno, F. J.; Mackie, A. R.; Mills, E. N. C. Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. *J. Agric. Food Chem.* **2005**, *53*, 9810-9816.

13. Martos, G.; Contreras, P.; Molina, E.; López-Fandiño, R. Egg white ovalbumin digestion mimicking physiological conditions. *J. Agric. Food Chem.* **2010**, *58*, 5640-5648.

14. Jiménez-Saiz, R.; Martos, G.; Carrillo, W.; López-Fandiño, R.; Molina, E. Susceptibility of lysozyme to in-vitro digestion and immunoreactivity of its digests. *Food Chem.* **2011**, *127*, 1719–1726.

15. Jiménez-Saiz, R.; Belloque, J.; Molina, E.; López-Fandiño, R. Human immunoglobulin E binding to heated and glycated ovalbumin and ovomucoid before and after in vitro digestion. *J. Agric. Food Chem.* **2011**, *59*, 10044-10051.

16. Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **2006**, *1*, 2856-2860.

17. Luque-Garcia, J. L. ; Zhou, G. ; Sun, T. T. ; Neubert, T. A. Use of nitrocellulose membranes for protein characterization by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **2006**, *78*, 5102-5108.

18. Harvey, D. J.; Wing, D. R.; Kuster, B.; Wilson, I. B. H. Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 564-571.

19. Hirose, J.; Kitabatake, N.; Kimura, A.; Narita, H. Recognition of native and/or thermally induced denatured forms of the major food allergen, ovomucoid, by human IgE and mouse monoclonal IgG antibodies. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2490-2497.

20. Cooke, S. K.; Sampson, H. A. Allergenic properties of ovomucoid in man. *J. Immuno.* **1997**, *59*, 2026-2032.

21. Zhang, J. W.; Mine, Y. Characterization of ovomucoid allergenic IgE epitopes using egg allergenic patients' sera. *Abstracts of Papers of the American Chemical Society* **1998**, *216*, U48-U48.

22. Amoresano, A.; Brancaccio, A.; Andolfo, A.; Perduca, M.; Monaco, H. L.; Marino, G. The carbohydrates of the isoforms of three avian riboflavin-binding proteins. *Eur. J. Biochem.* **1999**, *263*, 849-858.

23. Subramanian, S.; Adiga, P. R. Identification and mapping of linear antigenic determinants of chicken riboflavin carrier protein. *BBA: Prot. Struc. Mol. Enzymol.* **1998**, 1429, 74-82.

24. Subramanian, S.; Andal, S.; Karande, A. A.; Adiga, P. R. Epitope mapping of specificity of T-helper sites in tour major antigenic peptides of chicken riboflavin carrier protein in outbred rats. *Biochem. Biophys. Res. Comm.* **2003**, *311*, 11-16.

FIGURE CAPTIONS

Figure 1. RP-HPLC chromatograms of RfBP before digestion (T0), after 60 min of gastric digestion (G60), and after 30 (D30) and 60 min (D60) of the subsequent duodenal digestion. BS: bile salts.

Figure 2. SDS-PAGE of RfBP gastroduodenal digests under reducing (lanes 1-6) and non-reducing (lanes 7-12) conditions. Lanes **1, 7:** native RBP; **2, 8:** before digestion (T0); **3, 9:** 60 min gastric digest (G60); **4, 10:** 30 min gastroduodenal digest (D30); **5, 11:** 60 min gastroduodenal digest (D60); **6, 12** (24 h gastric digest).

Figure 3. IgE Inhibition ELISA of RfBP with three pooled sera from egg allergic patients. IgE binding of native RfBP (N), and 60 min gastric (G60) and 30 and 60 min gastroduodenal (D30 and D60) digests. Results are represented as the mean value (n=3). Error bars indicate the standard error of the mean.

Figure 4. SDS-PAGE (**A**) and immunoblot with allergic patients' pooled sera (**B**) of RfBP digests. Lanes **1:** before digestion (T0); **2:** 60 min gastric digest (G60); **3:** 30 min subsequent duodenal digest (D30); **4:** 24 h gastric digest.

Figure 5. Peptides identified by MALDI-TOF/TOF mass spectrometry after in-gel tryptic digestion of immunoreactive RfBP fragments R1 and R2 shown in Figure 4. **A)** Ion mass of detected peptides, their sequence and location in the protein. C* indicates carbamidomethylated cysteine. **B)** Primary sequence of mature RfBP. Bold and underlined residues correspond to peptides detected in the R2 and R1 fragments respectively.

TOC GRAPHIC

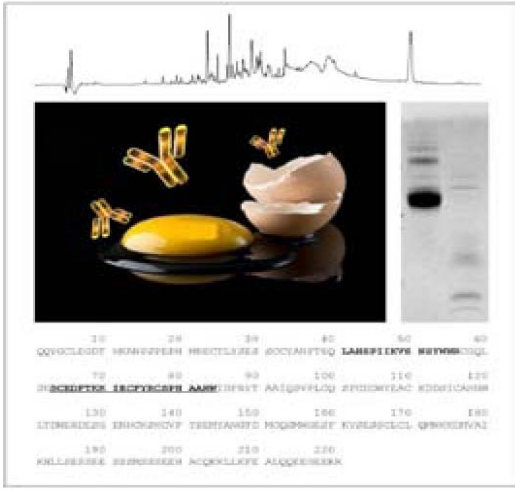


Figure 1

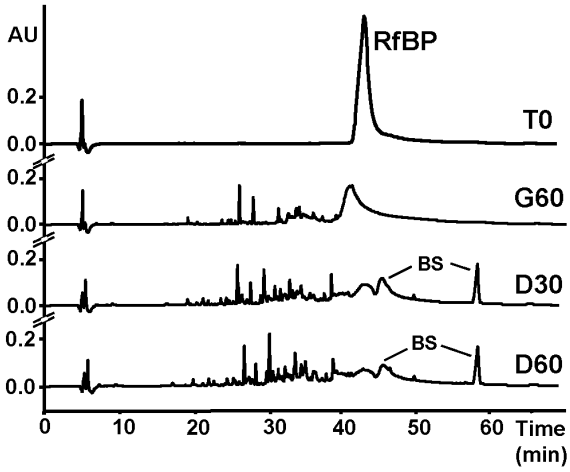


Figure 2

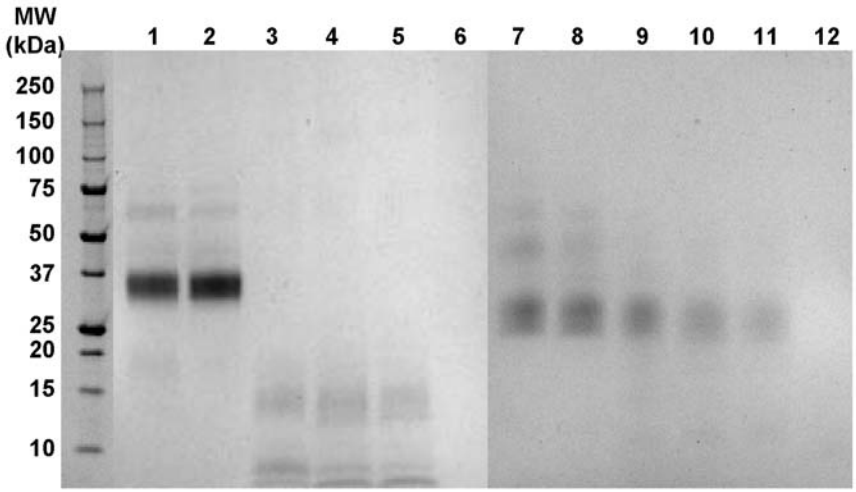


Figure 3

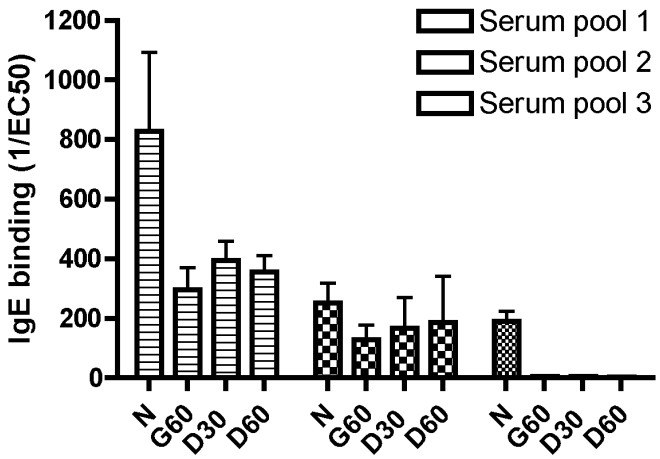
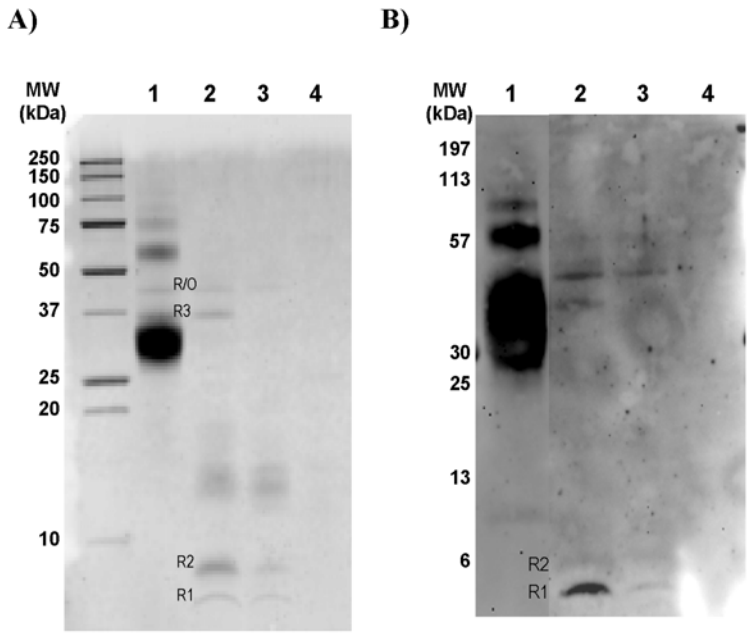

IgE binding (1/EC50)
1200
1000
800
600
400
200
0
Serum pool 1
Serum pool 2
Serum pool 3
N
G60
D30
D60
N
G60
D30
D60
N
G60
D30
D60

Figure 4


A)
MW (kDa)
1
2
3
4
250
150
100
75
50
37
25
20
10
R/O
R3
R2
R1
B)
MW (kDa)
1
2
3
4
197
113
57
30
25
13
6
R2
R1

A)

Fragment	Tryptic peptide (Ion Mass)	Protein residues	Sequence
R2 (5.1 kDa)			
	878.4	41-48	LAHSPIIK
	1025.5	49-56	VSNSYWNR
	1882.8	63-66	SC*EDFTKKIEC*F
	1015.5	70-76	KIEC*FYR
	781.4 (798-17)	77-83	C*SPHAAR
	967.4 (984-17)	77-84	C*SPHAARW
R1 (3.9 kDa)			
	1563.7	63-74	SC*EDFTKKIEC*F
	1882.8	63-76	SC*EDFTKKIEC*FYR
	781.4 (798-17)	77-83	C*SPHAAR
	967.4 (984-17)	77-84	C*SPHAARW

Figure 5

B)

```
        10         20         30         40         50         60
QQYGCLEGDT HKANPSPEPN MHECTLYSES SCCYANFTEQ LAHSPIIKVS NSYWNRCGQL

        70         80         90        100        110        120
SKSCEDFTKK IECFYRCSPH AARWIDPRYT AAIQSVPLCQ SFCDDWYEAC KDDSICAHNW

       130        140        150        160        170        180
LTDWERDESG ENHCKSKCVP YSEMYANGTD MCQSMWGESF KVSESSCLCL QMNKKDMVAI

       190        200        210        220
KHLLSESSEE SSSMSSSEEH ACQKKLLKFE ALQQEEGEERR
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Mechanisms underlying differential food allergy response to heated egg

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Mechanisms underlying differential food allergy response to heated egg

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Background: Egg white proteins are usually subjected to heating, making them edible for the majority of children with egg allergy.
Objective: We sought to investigate the underlying mechanisms responsible for the reduced allergenicity displayed by heat-treated egg white allergens.
Methods: C3H/HeJ mice were orally sensitized with ovalbumin (OVA) or ovomucoid and challenged with native or heated proteins to evaluate their allergenicity. Immunoreactivity was assessed by immunoblotting using sera from children with egg allergy. *In vitro* gastrointestinal digestion of native and heated OVA and ovomucoid was studied by SDS-PAGE and liquid chromatography. Intestinal uptake of intact native and heated OVA and ovomucoid by human intestinal epithelial (Caco-2) cells was investigated. Rat basophil leukemia cells passively sensitized with mouse serum and human basophils passively sensitized with serum from children with egg allergy were used to assess the effector cell activation by heated, digested, and transported OVA and ovomucoid.
Results: Heated OVA and ovomucoid did not induce symptoms of anaphylaxis in sensitized mice when administered orally. Heating did not completely destroy IgE-binding capacity of OVA or ovomucoid but enhanced *in vitro* digestibility of OVA. Digestion of both OVA and ovomucoid diminished mediator release in rat basophil leukemia assay and basophil activation. Heating of allergens prevented transport across human intestinal epithelial cells in a form capable of triggering basophil activation or T-cell activation.
Conclusion: Heat treatment reduces allergenicity of OVA and ovomucoid. This is partially a result of the enhanced gastrointestinal digestibility of heated OVA and the inability of heated OVA or ovomucoid to be absorbed in a form capable of triggering basophils. (J Allergy Clin Immunol 2011;127:990-7.)

Key words: *Egg allergy, ovalbumin, ovomucoid, heat treatment, heating, gastrointestinal digestion, antigen absorption, mice oral sensitization, anaphylaxis, basophil activation, passive sensitization*

Food processing and gastrointestinal degradation are fundamentally important for food protein allergenicity. Numerous reports[1-6] have addressed the effect of thermal and nonthermal processing on the final food allergenicity, which can be either enhanced or reduced depending on the particular allergen. Moreover, structural stability under the extreme degradative environment found within the gastrointestinal tract is often a requisite for a protein to elicit an allergic response.[7,8]

Food processing is of particular relevance for egg white allergens, because egg proteins are usually subjected to heat treatment such as boiling or baking and are likely to undergo important structural changes affecting their secondary and tertiary structure. It has been reported that approximately 70% of children with egg allergy tolerated baked egg ingestion.[9-12]

It is usually argued that heating induces protein denaturation, leading to the loss of conformational epitopes, suggesting that heated egg-tolerant children would present IgE antibodies mostly against conformational epitopes.[11,13] Heat-resistant proteins like ovomucoid, the dominant egg white allergen, can retain both linear and conformational epitopes on heating. However, ovomucoid-specific IgE levels were found to be poorly predictive of heated egg reactivity in a study enrolling 117 subjects with egg allergy.[12] Heat-induced aggregation of milk allergens was shown to prevent their absorption through enterocytes and subsequent onset of allergic symptoms in mice,[14] pointing at an additional explanation for tolerance to heated allergens.

These collective data suggest that extensive heating diminishes the allergenicity of egg white proteins, although the underlying mechanisms remain elusive. We sought to investigate the factors behind the reduced allergenicity displayed by the 2 major egg white allergens, ovalbumin (OVA) and ovomucoid, when they are subjected to heat treatment. We used *in vivo* and *in vitro* methods to compare digestion resistance, intestinal transport, and effector cell–triggering capacity of native and heated egg white proteins.

METHODS

Heating of OVA and ovomucoid

Ovalbumin (grade VI, 99% purity; Sigma, St Louis, Mo) and ovomucoid (trypsin inhibitor from chicken egg white, type III-O, free of ovoinhibitor;

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Abbreviations used
Bis-Tris: Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
DIG: Digoxigenin-3-0-succinyl-ϵ-aminocaproic acid-N-hydroxy-succinimide ester
kU_A/L: Kilo units of antibody per liter
MLN: Mesenteric lymph node
NHR: β-N-acetylhexosaminidase release
OVA: Ovalbumin
PP: Peyer patch
RBL: Rat basophil leukemia

Sigma) were dissolved as required for the different assays and heated in a boiling water bath for 30 minutes.

In vitro digestion of OVA and ovomucoid

Gastric digestion. OVA and ovomucoid were dissolved in simulated gastric fluid (35 mmol/L NaCl) at pH 2, preheated for 15 minutes at 37°C, and subjected to an *in vitro* gastric digestion with porcine pepsin (Enzyme Commission number 3.4.23.1, 3440 U/mg; Sigma) at an enzyme:substrate ratio of 1:20, wt/wt (172 U/mg). The reaction was stopped after 60 minutes with 1 mol/L $NaHCO_3$, for a final protein concentration of 5 mg/mL and pH 7.

Duodenal digestion. The starting material were gastric digests adjusted to pH 7 by adding 1 mol/L $CaCl_2$, 0.25 mol/L Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane (Bis-Tris), pH 6.5, and a 0.125-mol/L bile salt mixture containing equimolar quantities of sodium taurocholate (Sigma) and sodium glycodeoxycholate (Sigma). After preheating at 37°C for 15 minutes, porcine pancreatic lipase (EC 232-619-9; Sigma), colipase (EC 259-490-1; Sigma) and a commercial pancreatic mix, Corolase PP (AB Enzymes GmbH, Darmstadt, Germany) prepared in 35 mmol/L NaCl adjusted to pH 7, were added to the duodenal mix. The final composition of the mixture was 4.15 mg/mL OVA/ovomucoid, 6.15 mmol/L each bile salt, 20.3 mmol/L Bis-Tris, and 7.6 mmol/L $CaCl_2$, and the enzymes referred to the quantity of protein were 28.9 U/mg lipase, Corolase PP (enzyme:substrate ratio of 1:25, wt/wt) and colipase (enzyme:substrate ratio 1:895 wt/wt).

Digoxigenin labeling of egg white proteins

Proteins were incubated with digoxigenin-3-0-succinyl-ϵ-aminocaproic acid-N-hydroxy-succinimide ester (DIG; Roche Diagnostics, Indianapolis, Ind) for 2 hours at room temperature under constant shaking. Free DIG was eluted with PBS through a Sephadex PD-10 Column (Amersham Biosciences, Uppsala, Sweden).

RP-HPLC

Proteins and the corresponding hydrolysates at 4.15 mg/mL were separated in a Hi-Pore RP-318 (250 × 4.6 mm internal diameter) column (Bio-Rad, Richmond, Calif) in a Waters 600 HPLC (Waters Corp, Milford, Mass). The samples were eluted with 0.37% (vol/vol) trifluoroacetic acid in double-distilled water as solvent A and 0.27% (vol/vol) trifluoroacetic acid in acetonitrile as solvent B at 1 mL/min and 220 nm. Data were processed with Empower 2 Software (Waters Corp).

SDS-PAGE

Proteins were separated by SDS-PAGE (NuPAGE 4% to 12%, 15 wells; Invitrogen, Carlsbad, Calif) per the manufacturer's instructions; 6 μg protein was loaded per well. Proteins were transferred onto Immobilon-P PVDF membranes (Millipore, Bedford, Mass) and probed with sera from children with egg allergy.

Serum samples

A serum pool was made of equal parts of serum from 8 heated egg–reactive children with egg allergy as documented with an oral challenge. Levels of specific IgE antibodies were measured with UniCAP (Phadia US, Portage, Mich), lower limit of detection, 0.35, and upper limit of detection, 100 kilo units of antibody per liter (kU_A/L). Pool specific IgE levels were as follows: egg white, 12.8; OVA, 14.0; and ovomucoid, 13.9 k_AU/L.

Immunoblotting

Immunoblots for detection of IgE binding were performed with native and heated OVA and ovomucoid. Membranes were incubated with an egg-allergic serum pool 1:10 dilution in PBS containing 0.05% Tween 20, 1% BSA, and 10% normal goat serum for 60 minutes. PBS-rinsed membranes were incubated with ^{125}I-goat antihuman IgE (DiaMed, Windham, Me) for 1 hour, washed, and exposed to Kodak BioMax MS Film (Carestream Health Inc, Rochester, NY) for 1 to 12 days. As a negative control, serum from a non-atopic adult was used.

Sensitization and oral challenge of mice

Five-week-old female C3H/HeJ mice (NCI, Fredrick, Md) were sensitized orally with 1 mg native OVA (n = 15) or ovomucoid (n = 24) in 0.2 mol/L bicarbonate buffer plus 10 μg cholera toxin (List Biologicals, Campbell, Calif) per week for 6 weeks. On week 7, all sensitized mice were orally challenged with either native or heated OVA and ovomucoid. Five OVA-sensitized mice were challenged 1 week apart with both heated and unheated OVA. Total doses of 30 and 42 mg OVA and ovomucoid, respectively, were administered in 2 increments, 15 minutes apart. If no symptoms were observed, the mice were then challenged with 100 μg allergen intraperitoneally. Animal studies were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine.

Anaphylaxis assessment

Symptoms were scored as previously published.[14] Rectal temperature (World Precision Instruments, Sarasota, Fla) was measured as a further assessment of anaphylaxis severity.

Measurement of antigen-specific IgE

Mouse OVA-specific and ovomucoid-specific IgE was quantified by ELISA. A 96-well plate was coated overnight at 4°C with rat antimouse IgE antibody (BD Biosciences, San Jose, Calif), then blocked with 10% normal mouse serum, 1% BSA in PBS 0.05%Tween. After incubation with serum from sensitized mice, DIG-conjugated OVA or ovomucoid was added. Finally, horseradish peroxidase–labeled anti-DIG antibody (fragment antigen-binding fragments; Roche Diagnostics) was incubated with tetramethylbenzidine (BD Biosciences) as a substrate. The reaction was stopped with 1.2 mol/L sulfuric acid, and absorbance was measured at 450 nm.

In vitro cytokine responses

Splenocytes were plated at a density of 5×10^6 cells/mL in 24-well cell tissue culture plates (Nalge Nunc, Naperville, Ill) with 50 μg/mL OVA and ovomucoid proteins, respectively, or medium alone (RPMI 1640) in 10% FCS for 72 hours at 37°C in 5% CO_2. Cytokines in culture supernatants were measured by ELISA (eBiosciences, San Diego, Calif).

Mediator release assay

Rat basophil leukemia (RBL) cells (RBL-2H3; kind gift of Dr Stefan Vieths) were cultured in Eagle minimal essential medium with 10% FCS, and the assay was performed as published.[15] Briefly, RBL cells (at 3×10^6 cells/mL) were incubated with serum at a final dilution of 1:60 at 37°C in 5% CO_2 overnight in 96-well tissue culture plates (BD Falcon; BD, Bedford, Mass). Both OVA-sensitized and ovomucoid-sensitized mouse pool sera were used. Sensitized cells were stimulated with 100 μL per well of the dilutions of allergens. Rat antimouse IgE (Pharmingen) was used as a positive control for IgE-mediated degranulation; RBL cells were lysed with 1% Triton X-100 (Sigma) for total release. β-N-acetylhexosaminidase release (NHR) on

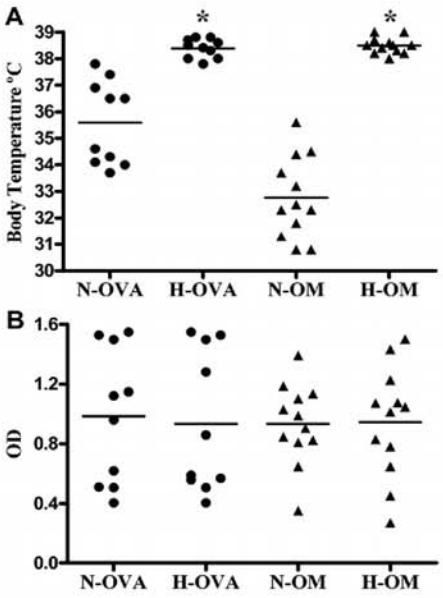

FIG 1. **A,** Mice body temperature on oral challenge with native *(N)* or heated *(H)* OVA or ovomucoid *(OM)*. **B,** Serum OVA-specific and OM-specific IgE levels of the different mice groups before challenge. OD values correspond to 1/100 serum dilution. *Statistically significant differences between native and heated challenged groups; $P < .001$.

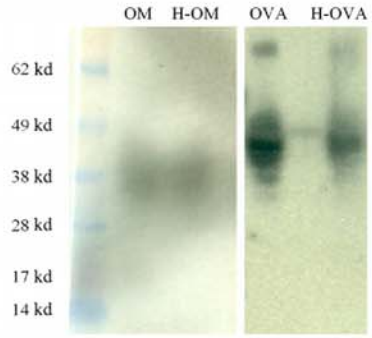

FIG 2. Immunoblotting of unheated or heated *(H)* ovomucoid *(OM)* and OVA by using pooled sera from heated egg–reactive children.

stimulation with allergen was determined. Spontaneous release (stimulation with buffer only) was subtracted from the allergen-induced NHR at every dilution point. Values were expressed as percentage of the total release, and the protein concentration that gave 50% of the maximum NHR (inhibitory concentration$_{50}$) was determined.[16,17]

Transcytosis studies

Caco-2 cells (clone C2Bbe1; American Type Culture Collection, Rockville, Md) were seeded on 0.4-μm Transwell filter (Cole-Parmer, Vernon Hills, Ill) inserts at a concentration of 5×10^5 cells/mL. After 1 week, transepithelial resistance was checked by ohmmeter (World Precision Instruments) and monolayers used if resistance was greater than 300 Ω. Then fluorescein isothiocyanate–labeled native and heated OVA and ovomucoid were added in triplicate to the apical side (0.5 mL at 0.5 mg/mL) and incubated at 37°C/5% CO_2 for 20 hours. Samples from the basolateral side were collected after the incubation period.

OVA-specific T-cell activation

$CD4^+$ T cells were isolated from spleens and lymph nodes of DO11.10 mice by negative selection (StemCell, Vancouver, British Columbia, Canada) and labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen). Cells 3 to 5×10^6 were injected into naive BALB/c recipients intravenously. The next day, mice were fed 25 mg native or heated OVA in a total volume of 1 mL, given as 2 doses 1 hour apart. After 72 hours, mice were euthanized, and mesenteric lymph node (MLN) and Peyer patch (PP) cells were isolated. Cells were stained with antibodies against CD4 and the DO11 TCR (KJ1-26), and dead cells identified and excluded with a violet live/dead staining kit (Invitrogen). Cells were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed by using FlowJo software (Treestar, Ashland, Ore).

Stripping and passive sensitization of basophils from donors without allergy

PBMCs were isolated by using Ficoll from egg-tolerant adult donors. For stripping of bound IgE,[18] the PBMC pellet was resuspended in 3 mL lactic acid solution (13.4 mmol/L lactate, 140 mmol/L NaCl, 5 mmol/L KCl, pH 3.9) and incubated on ice for 5 minutes. Human serum albumin 0.5%, 7 mL (Sigma), in RPMI medium with glutamine (Fisher Scientific, Pittsburgh, Pa) was added, and the solution was neutralized with 15 μL 12% TRIS. After centrifuging and washing the pellet with human serum albumin/RPMI, it was resuspended in 1/100 of the original blood volume. Stripped PBMCs were placed in a 96-well U-bottom plate and incubated for 1 hour at 37°C with 1:2 diluted serum (20 μL PBMCs + 20 μL diluted serum) from a pool of 3 children with egg allergy (OVA-specific and ovomucoid-specific IgE levels were 63.4 and 29.8 kU_A/L, respectively).

Basophil activation assay

PBMCs sensitized as described were incubated with basophil stimulation buffer RPMI with IL-3 (25 μg/mL; R&D Systems, Minneapolis, Minn) as a negative control or with anti-IgE antibody (25 μg/mL; Bethyl Laboratories, Montgomery, Tex) as a positive control. Samples (4 μg/mL) were incubated with native, heated, and/or digested OVA and ovomucoid or with basolateral supernatants from transcytosis experiments at a 1:5 dilution. The reaction was stopped after 30 minutes with EDTA in cold PBS. Cells were stained for CD63, CD123, HLA-DR (BD Biosciences), and CD203c (Beckman Coulter), and fixed with Fluorescence-Activated Cell Sorting Lysing Solution (BD Biosciences). Cells were acquired as described.

Statistical analysis

Differences between mice groups were analyzed by independent *t* test. Donors' basophil stimulation percentages for heated and digested samples were compared by ratio-paired *t* test. *P* values below .05 were considered significant. Statistical analysis was performed by using GraphPad Prism software (La Jolla, Calif).

RESULTS

Heated OVA and ovomucoid do not induce anaphylaxis in an animal model

Mice were sensitized to native OVA (n = 15) or ovomucoid (n = 24) with cholera toxin. On oral challenge with the native proteins, ovomucoid-sensitized mice developed higher anaphylaxis scores and lower body temperature than OVA-sensitized mice ($P < .001$), confirming that ovomucoid is a stronger allergen. After oral challenge with native ovomucoid and native OVA, all mice presented symptoms of anaphylaxis. In contrast, mice challenged with the heated allergens did not develop anaphylaxis, as assessed by body temperature measurement (Fig 1) and symptom score (a median score of 4 and 2 for native ovomucoid and OVA, respectively, compared with a median score of 0 for both heated

J ALLERGY CLIN IMMUNOL
VOLUME 127, NUMBER 4

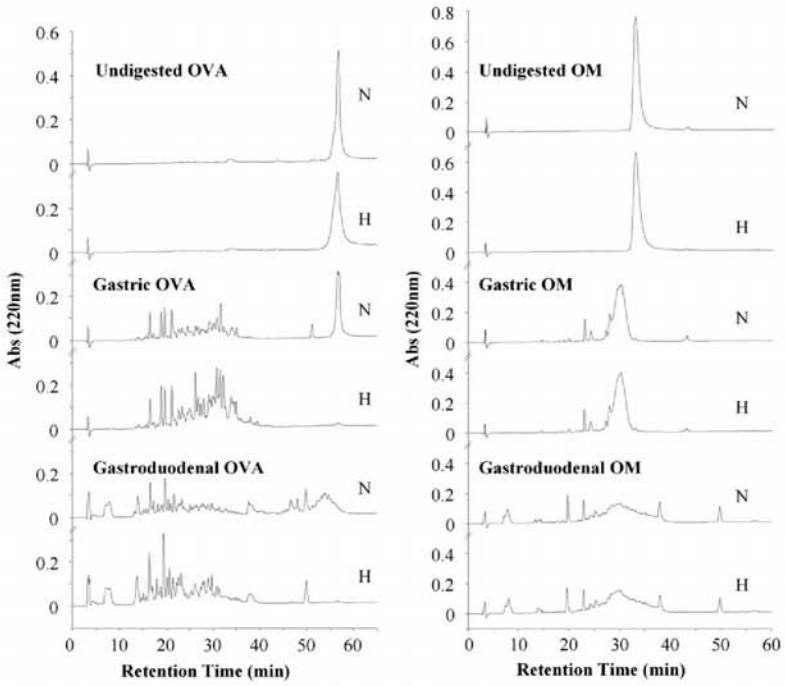

FIG 3. RP-HPLC chromatograms corresponding to native *(N)* and heated *(H)* OVA and ovomucoid *(OM)* undigested and subjected to gastric and gastroduodenal digestion.

ovomucoid and OVA; $P < .01$). However, when mice were subsequently systemically (intraperitoneally) challenged with the heated allergens, the majority of them (3/5 for heated OVA and 6/7 for heated ovomucoid) showed mild symptoms and a drop in body temperature. Serum specific IgE levels were similar in all mice groups (Fig 1, *B*), as were OVA and ovomucoid-induced IL-13 and IFN-γ production from spleen cells (see this article's Fig E1 in the Online Repository at www.jacionline.org).

Heat treatment does not completely destroy OVA and ovomucoid IgE-binding epitopes

Native and heated OVA and ovomucoid were immunolabeled with a serum pool from extensively heated egg–reactive children to assess the binding of serum IgE. There was no appreciable decrease in binding to heated ovomucoid and some decrease in binding to heated OVA. Both OVA and ovomucoid showed strong binding regardless of heat treatment (Fig 2), suggesting the persistence of linear epitopes recognized by IgE.

Heat treatment makes OVA more susceptible to digestion, whereas it does not affect ovomucoid

We used an *in vitro* digestion model to evaluate the effect of heating on the final outcome of OVA and ovomucoid. Samples were collected after 60 minutes (gastric phase) and 120 minutes (duodenal phase) and analyzed by RP-HPLC (Fig 3). Native OVA was poorly hydrolyzed by pepsin. However, heated OVA became completely hydrolyzed after 60 minutes. In contrast, heat treatment did not alter ovomucoid susceptibility to either pepsin or duodenal enzymes, producing similar digestion profiles where ovomucoid was quickly digested, giving rise to fragments of lower molecular weight.

Heat treatment and digestion significantly reduce the capacity of OVA and ovomucoid to trigger basophil activation and degranulation in the RBL-based mediator release assay

We aimed to determine whether heated proteins or the peptides generated after digestion were able to activate basophils comparably to native proteins. We isolated basophils from 3 egg-tolerant adult donors and passively sensitized them with pooled sera from children with allergy. Passively sensitized basophils were stimulated with either native or heated OVA and ovomucoid, as well as the corresponding gastroduodenally digested proteins. The population of activated basophils analyzed by flow cytometry is shown in Fig 4. Challenge of basophils with native OVA or ovomucoid induced basophil activation as measured by upregulation of CD63. OVA exhibited a significant reduction in basophil activation only when it was both heated and digested. Ovomucoid stimulation was reduced after digestion regardless of heating, whereas heating alone did not reduce basophil activation.

We verified these results by using RBL cells passively sensitized with serum from ovomucoid-sensitized mice (Fig 5). Digested ovomucoid gave reduced NHR compared with undigested ovomucoid, regardless of heating status. The IC_{50} of ovomucoid was approximately 100-fold higher on digestion, 10 μg/mL for digested ovomucoid versus 0.1 μg/mL for undigested ovomucoid. These data suggest that heating does not affect ovomucoid basophil activation capacity and mediator release from RBLs, but proteolysis by gastroduodenal enzymes greatly decreases it.

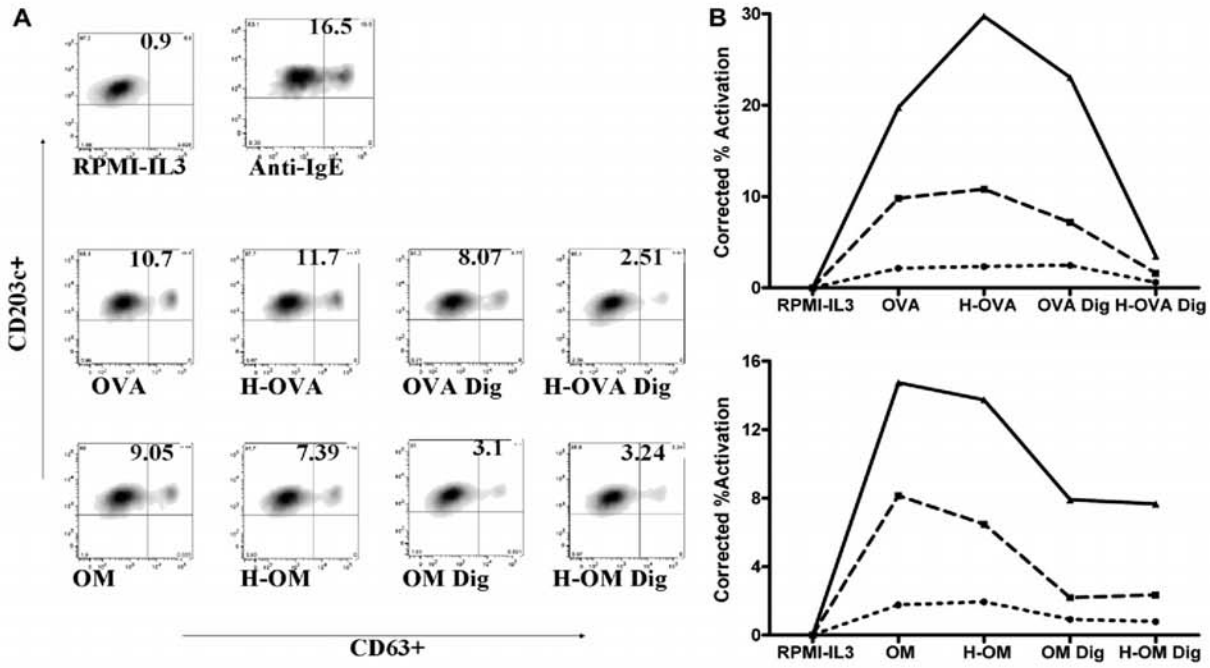

FIG 4. Percentage of activated basophils ($CD63^+CD123^+CD203c^+HLA\text{-}DR^{low}$) on stimulation with native, heated *(H)*, and/or digested *(Dig)* OVA or ovomucoid *(OM)*. **A,** Detailed activated basophil population from 1 donor. **B,** Activation percentages from 3 donors.

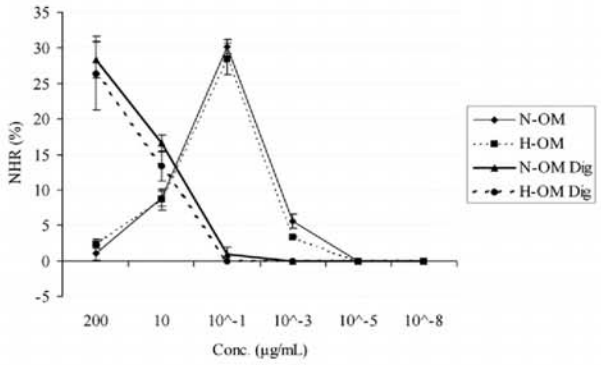

FIG 5. NHR of RBL cells passively sensitized with ovomucoid *(OM)*–reactive mice's sera and stimulated with different concentrations *(Conc.)* of undigested/digested native *(N)* and heated *(H)* OM. NHR release by negative controls (digestive enzymes, buffer) was not significantly different from spontaneous release, which was subtracted in the final graph. Positive control, anti-IgE stimulation, was 60%.

Heating prevents transcytosis of intact allergen across intestinal epithelial cells and PPs

Food allergens must cross the intestinal epithelial barrier before activating allergic effector cells. To model this, native and heated OVA and ovomucoid were added to the apical side of a polarized monolayer of Caco-2 human intestinal epithelial cells grown in Transwells. Supernatants were collected from the basolateral compartment after an overnight culture, and we then evaluated the basophil activation by the transcytosed proteins (Fig 6). Native OVA and ovomucoid were readily transported across Caco-2 monolayers and triggered significant basophil activation. In contrast, heated OVA and heated ovomucoid triggered markedly decreased basophil activation.

To assess the impact of heating on gastrointestinal uptake of antigen *in vivo*, we assessed antigen-specific T-cell activation in the gastrointestinal-associated lymphoid tissue by using an adoptive transfer model. CFSE-labeled OVA-specific DO11.10 T cells were injected into naive BALB/c mice before oral feeding with native or heated OVA. Proliferation of DO11.10 T cells was assessed in the MLN and PP (Fig 7). Mice fed with native OVA presented extensive proliferation in both the MLN and the PP compared with unfed mice. In contrast, mice fed with heated OVA had minimal T-cell proliferation in either the MLN or the PP. Thus, in addition to facilitating digestion of OVA, heating of OVA and ovomucoid abrogates their intestinal absorption in an intact form capable of triggering effector cells and T cells.

DISCUSSION

The *in vivo* murine model of egg-induced anaphylaxis used here reproduces the observation that the majority (over 70%) of children with egg allergy can tolerate extensively heated egg.[9,10,12,19] Mice were completely tolerant to heated egg delivered by the oral, but not the systemic, route. This suggests that heating may influence the handling of egg allergens in the gastrointestinal tract in addition to altering the conformation of IgE-binding epitopes. We showed that heating did not completely destroy epitopes recognized by IgE antibodies from children with egg allergy. This was true for IgE binding and for activation of basophils passively sensitized with serum from children with egg allergy or mice. Therefore, our goal was to determine why ingestion of heated OVA and ovomucoid did not cause symptoms despite the persistence of IgE-binding epitopes.

J ALLERGY CLIN IMMUNOL
VOLUME 127, NUMBER 4

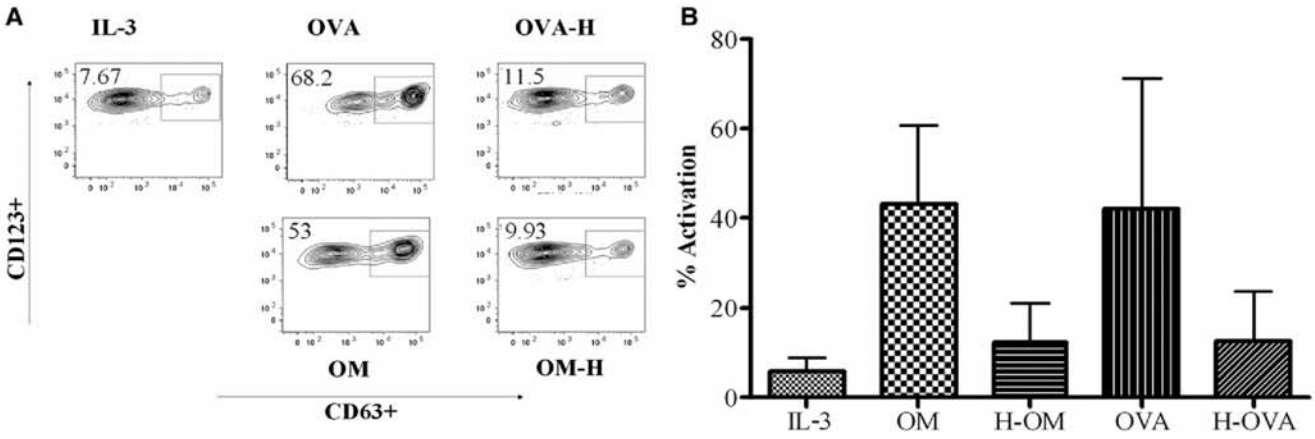

FIG 6. Percentage of activated basophils ($CD63^+CD123^+CD203c^+HLA\text{-}DR^{low}$) on stimulation with native or heated *(H)* OVA or ovomucoid *(OM)* transcytosed by Caco-2 cells. **A,** Detailed activated basophil population from 1 experiment. **B,** Mean activation percentages from 3 replicates. Error bars represent SD.

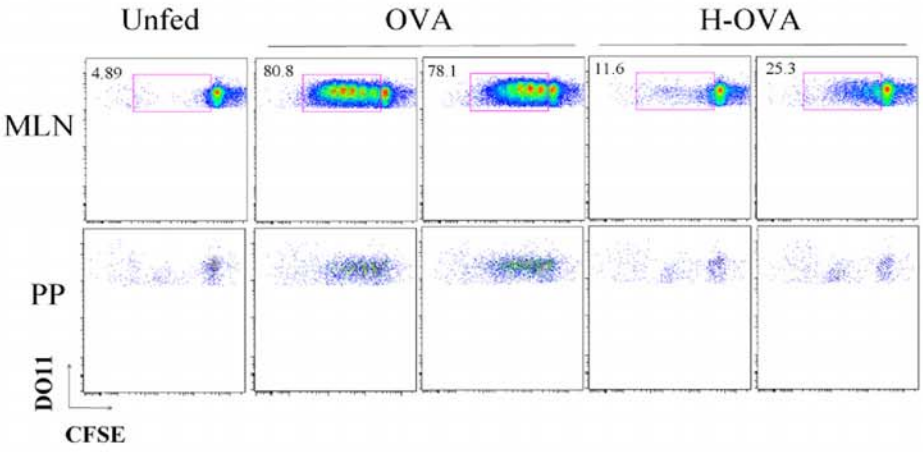

FIG 7. OVA-specific T-cell activation in the gastrointestinal lymphoid tissue. CFSE-labeled DO11.10 T cells were transferred to naive BALB/c mice before gavage feeding with 25 mg OVA or heated *(H)* OVA. After 72 hours, proliferation of DO11.10 T cells was assessed by flow cytometry by using cells isolated from MLN or PP. Data are shown from 2 individual OVA-fed or H-OVA–fed mice.

We confirmed that OVA changes its structure on heating and forms high-molecular-weight aggregates (observed by inability to enter SDS-PAGE gels; see this article's Fig E2 in the Online Repository at www.jacionline.org). In contrast, ovomucoid remains largely unaffected in the gel after heating, although there is strong evidence of an irreversibly heat-denatured form of ovomucoid with newly appearing specific IgE epitopes.[20]

For food allergens to trigger anaphylaxis, they must escape digestion and be absorbed in a sufficiently intact or immunologically active form across the epithelial barrier.[21-23] We examined how heating of egg white proteins altered each of these factors. In globular proteins like OVA, heating is known to induce a partially folded molten globule structure responsible for their gelling and emulsifying properties.[24] This structure is also favored at the low pH of the gastric phase,[25] and it is differentiated from the native state by the absence of close packing throughout the molecule.[26] It has been reported that OVA's high resistance to pepsin[27] is lost when heated.[28] Heat treatment would therefore facilitate the access of gastrointestinal proteases to potential cleavage sites, resulting in a complete degradation of OVA. In contrast, the ovomucoid structure made up of 9 disulfide bonds and 25% carbohydrate content is likely responsible for its high thermal stability and limited denaturation.[29] This could explain the unchanged enzymatic susceptibility of ovomucoid observed under gastrointestinal conditions.

Resistance to digestion is a common characteristic shared by many allergens. Sedimentation coefficient equal to approximately 2 albumins from mustard,[30] Brazil nuts,[31] or sesame seeds[32] and lipid transfer proteins from grape[33] or cherry[34] are only some examples of highly stable allergens. In addition, there are allergens that, despite being rapidly digested, give raise to stable fragments which retain allergenicity, as in the case of Ara h 1,[35-38] the major peanut allergen. Impaired gastrointestinal function entails an increased risk of systemic absorption of food antigens. Hindered degradation of codfish allergens occurring at increased gastric pH conditions results in the maintenance of biological activity even after 2 hours of digestion, as evidenced by their histamine-

releasing capacity.[7] Incomplete digestion of kiwifruit at an increased gastric pH has also been reported,[39] and the use of antacid medication has been related to sensitization to dietary proteins because of incomplete enzymatic degradation.[40] Hence, food processing such as heat treatment, which facilitates OVA degradation within the gastrointestinal tract, would have a beneficial effect on preventing adverse reactions in individuals with allergy.

We next assessed the effect of heating on transcytosis of intact egg white allergens across human intestinal epithelium. Heating of OVA and ovomucoid completely abrogated the delivery of immunologically intact forms of allergen across the epithelial monolayer. Heat treatment of milk proteins has been shown to cause aggregation of the whey proteins, redirecting antigen uptake away from absorptive enterocytes to PPs. The immediate consequence of this pathway switch was the abrogation of anaphylactic response in mice, probably because aggregated antigens transported into PP bypass lamina propria mast cells or fail to reach the systemic circulation.[14] Here, however, the lack of proliferating OVA-specific CD4 T cells in the MLN or PP argues that OVA is not getting absorbed into the gut mucosa, and unlike the heated milk proteins does not seem to be getting taken up into the PP either. The aggregation of OVA was likely responsible for this blockade of absorption, whereas ovomucoid does not form aggregates when heated.[11] Moreover, gastrointestinal digestibility of ovomucoid was unaffected by heat treatment. However, the finding that heated ovomucoid was not transported across epithelial cells in a form capable of triggering basophils could indicate enhanced intracellular degradation as it crosses the monolayer of cells. In *ex vivo* studies of animal and human intestinal mucosa, only small amounts of intact food antigens were transcytosed (~0.1% of luminal concentration) by intestinal epithelial cells.[41] Large proteins taken up by intestinal epithelial cells were released in their basal pole either as immunogenic peptides (~40%) or fully degraded into amino acids (~50%) with only a minor fraction crossing the epithelium in their intact form.[42] We have shown that ovomucoid degradation by enzymes diminished its basophil activation capacity to a great extent, yet we did not address the additional impact of intracellular enzymes in enterocytes. In the case of ovomucoid, we hypothesize that heating could render it more susceptible to enterocytic intracellular enzymes generating nonallergenic peptides.

Another factor that might affect both the integrity of IgE-binding epitopes and gastrointestinal digestibility and absorption is interaction between heated egg white proteins and a complex food matrix.[6,43] A study by Kato et al[44] demonstrated a marked decrease in the solubility of ovomucoid when egg white was mixed with wheat flour and wheat gluten and then heated at 180°C for 10 minutes, mimicking the process of bread-making. Immunoblotting suggested that ovomucoid polymerized and formed high-molecular-weight complexes with gluten leading to aggregation and insolubilization of ovomucoid. This phenomenon might further decrease the accessibility to digestion and slow the absorption of intact allergenic particles, relevant to human studies.[12]

The use of heated proteins arises as an attractive strategy for oral immunotherapy. Our data confirm the relative safety of heated egg white proteins. However, it is important to remark that despite their diminished capacity to trigger effector cells, alteration of the allergen structure may either enhance sensitizing potential or abrogate tolerogenic capacity. Heat treatment of milk proteins was shown to promote sensitization in C3H/HeJ mice.[14] Boiling of egg white proteins abrogated the suppression of T_H2 responses in BALB/c mice receiving the antigens before sensitization.[45] Furthermore, glycated OVA as a result of the Maillard reaction during thermal processing was shown to induce enhanced activation of OVA-specific $CD4^+$ T cells on coculture with myeloid dendritic cells compared with native OVA and OVA processed without glucose.[46]

Our findings emphasize that food processing can fundamentally alter the ability of food protein allergens to trigger reactions not solely by interfering with their IgE binding, but by altering their degradation and absorption within the gastrointestinal tract.

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Clinical implications: Reduced allergenicity of heated egg white proteins partially resulting from altered digestion and absorption in the gastrointestinal tract may explain the clinical tolerance of extensively heated egg in the majority of children with egg allergy.

REFERENCES

1. Peyron S, Mouecoucou J, Fremont S, Sanchez C, Gontard N. Effects of heat treatment and pectin addition on beta-lactoglobulin allergenicity. J Agric Food Chem 2006;54:5643-50.
2. Poulsen OM, Hau J, Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. Clin Allergy 1987;17:449-58.
3. Bernhisel-Broadbent J, Strause D, Sampson HA. Fish hypersensitivity, II: clinical relevance of altered fish allergenicity caused by various preparation methods. J Allergy Clin Immunol 1992;90:622-9.
4. Fiocchi A, Restani P, Riva E, Restelli AR, Biasucci G, Galli CL, et al. Meat allergy, II–effects of food processing and enzymatic digestion on the allergenicity of bovine and ovine meats. J Am Coll Nutr 1995;14:245-50.
5. Restani P, Fiocchi A, Restelli AR, Velona T, Beretta B, Giovannini M, et al. Effect of technological treatments on digestibility and allergenicity of meat-based baby foods. J Am Coll Nutr 1997;16:376-82.
6. Thomas K, Herouet-Guicheney C, Ladics G, Bannon G, Cockburn A, Crevel R, et al. Evaluating the effect of food processing on the potential human allergenicity of novel proteins: international workshop report. Food Chem Toxicol 2007;45:1116-22.
7. Untersmayr E, Vestergaard H, Malling HJ, Jensen LB, Platzer MH, Boltz-Nitulescu G, et al. Incomplete digestion of codfish represents a risk factor for anaphylaxis in patients with allergy. J Allergy Clin Immunol 2007;119:711-7.
8. Breiteneder H, Mills EN. Molecular properties of food allergens. J Allergy Clin Immunol 2005;115:14-23; quiz 24.
9. Des Roches A, Nguyen M, Paradis L, Primeau MN, Singer S. Tolerance to cooked egg in an egg allergic population. Allergy 2006;61:900-1.
10. Konstantinou GN, Giavi S, Kalobatsou A, Vassilopoulou E, Douladiris N, Saxoni-Papageorgiou P, et al. Consumption of heat-treated egg by children allergic or sensitized to egg can affect the natural course of egg allergy: hypothesis-generating observations. J Allergy Clin Immunol 2008;122:414-5.
11. Urisu A, Ando H, Morita Y, Wada E, Yasaki T, Yamada K, et al. Allergenic activity of heated and ovomucoid-depleted egg white. J Allergy Clin Immunol 1997;100:171-6.
12. Lemon-Mule H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, Nowak-Wegrzyn A. Immunologic changes in children with egg allergy ingesting extensively heated egg. J Allergy Clin Immunol 2008;122:977-83, e1.
13. Cooke SK, Sampson HA. Allergenic properties of ovomucoid in man. J Immunol 1997;159:2026-32.
14. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy 2008;63:882-90.

15. Nowak-Wegrzyn AH, Bencharitiwong R, Schwarz J, David G, Eggleston P, Gergen PJ, et al. Mediator release assay for assessment of biological potency of German cockroach allergen extracts. J Allergy Clin Immunol 2009;123:949-55, e1.
16. Hoffmann A, Kaul S, Fotisch K, Danz N, Luttkopf D, Hatahet L, et al. Mediator release assays based on human and murine IgE: potential and limitations in allergen standardization. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M 2003;94:76-85, discussion 85-6.
17. Vieths S, Jankiewicz A, Aulepp H, Haustein D. Allergy to heated and processed foods. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M 1997;91: 250-62.
18. Pruzansky JJ, Grammer LC, Patterson R, Roberts M. Dissociation of IgE from receptors on human basophils, I: enhanced passive sensitization for histamine release. J Immunol 1983;131:1949-53.
19. Ando H, Moverare R, Kondo Y, Tsuge I, Tanaka A, Borres MP, et al. Utility of ovomucoid-specific IgE concentrations in predicting symptomatic egg allergy. J Allergy Clin Immunol 2008;122:583-8.
20. Hirose J, Kitabatake N, Kimura A, Narita H. Recognition of native and/or thermally induced denatured forms of the major food allergen, ovomucoid, by human IgE and mouse monoclonal IgG antibodies. Biosci Biotechnol Biochem 2004;68: 2490-7.
21. Taylor SL, Hefle SL. Will genetically modified foods be allergenic? J Allergy Clin Immunol 2001;107:765-71.
22. Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Crit Rev Food Sci Nutr 1996;36(suppl):S165-86.
23. Taylor SL. Protein allergenicity assessment of foods produced through agricultural biotechnology. Annu Rev Pharmacol Toxicol 2002;42:99-112.
24. Hirose M. Molten globule state of food proteins. Trends Food Sci Technol 1993;4: 48-51.
25. Koseki T, Kitabatake N, Doi E. Conformational changes in ovalbumin at acid pH. J Biochem 1988;103:425-30.
26. Kuwajima K. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. Proteins 1989;6:87-103.
27. Martos G, Contreras P, Molina E, Lopez-Fandino R. Egg white ovalbumin digestion mimicking physiological conditions. J Agric Food Chem 2010;58:5640-8.
28. Takagi K, Teshima R, Okunuki H, Sawada J. Comparative study of in vitro digestibility of food proteins and effect of preheating on the digestion. Biol Pharm Bull 2003;26:969-73.
29. Djurtoft R, Pedersen HS, Aabin B, Barkholt V. Studies of food allergens: soybean and egg proteins. Adv Exp Med Biol 1991;289:281-93.
30. Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion in vitro. Nat Biotechnol 1996;14:1269-73.
31. Moreno FJ, Mellon FA, Wickham MS, Bottrill AR, Mills EN. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. FEBS J 2005;272:341-52.
32. Moreno FJ, Maldonado BM, Wellner N, Mills EN. Thermostability and in vitro digestibility of a purified major allergen 2S albumin (Ses i 1) from white sesame seeds (Sesamum indicum L.). Biochim Biophys Acta 2005;1752:142-53.
33. Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, et al. Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. J Allergy Clin Immunol 2006;118:473-80.
34. Scheurer S, Lauer I, Foetisch K, San Miguel Moncin M, Retzek M, Hartz C, et al. Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. J Allergy Clin Immunol 2004;114:900-7.
35. Maleki SJ, Kopper RA, Shin DS, Park CW, Compadre CM, Sampson H, et al. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. J Immunol 2000;164:5844-9.
36. Eiwegger T, Rigby N, Mondoulet L, Bernard H, Krauth MT, Boehm A, et al. Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential. Clin Exp Allergy 2006;36:1281-8.
37. van Boxtel EL, Koppelman SJ, van den Broek LA, Gruppen H. Determination of pepsin-susceptible and pepsin-resistant epitopes in native and heat-treated peanut allergen Ara h 1. J Agric Food Chem 2008;56:2223-30.
38. Koppelman SJ, Hefle SL, Taylor SL, de Jong GA. Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. Mol Nutr Food Res 2010;54: 1711-21.
39. Lucas JS, Cochrane SA, Warner JO, Hourihane JO. The effect of digestion and pH on the allergenicity of kiwifruit proteins. Pediatr Allergy Immunol 2008;19:392-8.
40. Untersmayr E, Scholl I, Swoboda I, Beil WJ, Forster-Waldl E, Walter F, et al. Antacid medication inhibits digestion of dietary proteins and causes food allergy: a fish allergy model in BALB/c mice. J Allergy Clin Immunol 2003;112:616-23.
41. Heyman M, Desjeux JF. Antigen handling by intestinal epithelial cells. In: Kaiserlian D, ed. Antigen Presentation by Intestinal Epithelial Cells. London: Landes; 1996. p.p. 1-19.
42. Heyman M, Ducroc R, Desjeux JF, Morgat JL. Horseradish peroxidase transport across adult rabbit jejunum in vitro. Am J Physiol 1982;242:G558-64.
43. Teuber SS. Hypothesis: the protein body effect and other aspects of food matrix effects. Ann N Y Acad Sci 2002;964:111-6.
44. Kato Y, Watanabe H, Matsuda T. Ovomucoid rendered insoluble by heating with wheat gluten but not with milk casein. Biosci Biotechnol Biochem 2000;64: 198-201.
45. Peng HJ, Chang ZN, Tsai LC, Su SN, Shen HD, Chang CH. Heat denaturation of egg-white proteins abrogates the induction of oral tolerance of specific Th2 immune responses in mice. Scand J Immunol 1998;48:491-6.
46. Ilchmann A, Burgdorf S, Scheuer S, Waibler Z, Nagai R, Wellner A, et al. Glycation of a food allergen by the Maillard reaction enhances its T-cell immunogenicity: role of macrophage scavenger receptor class A type I and II. J Allergy Clin Immunol 2010;125:175-83.

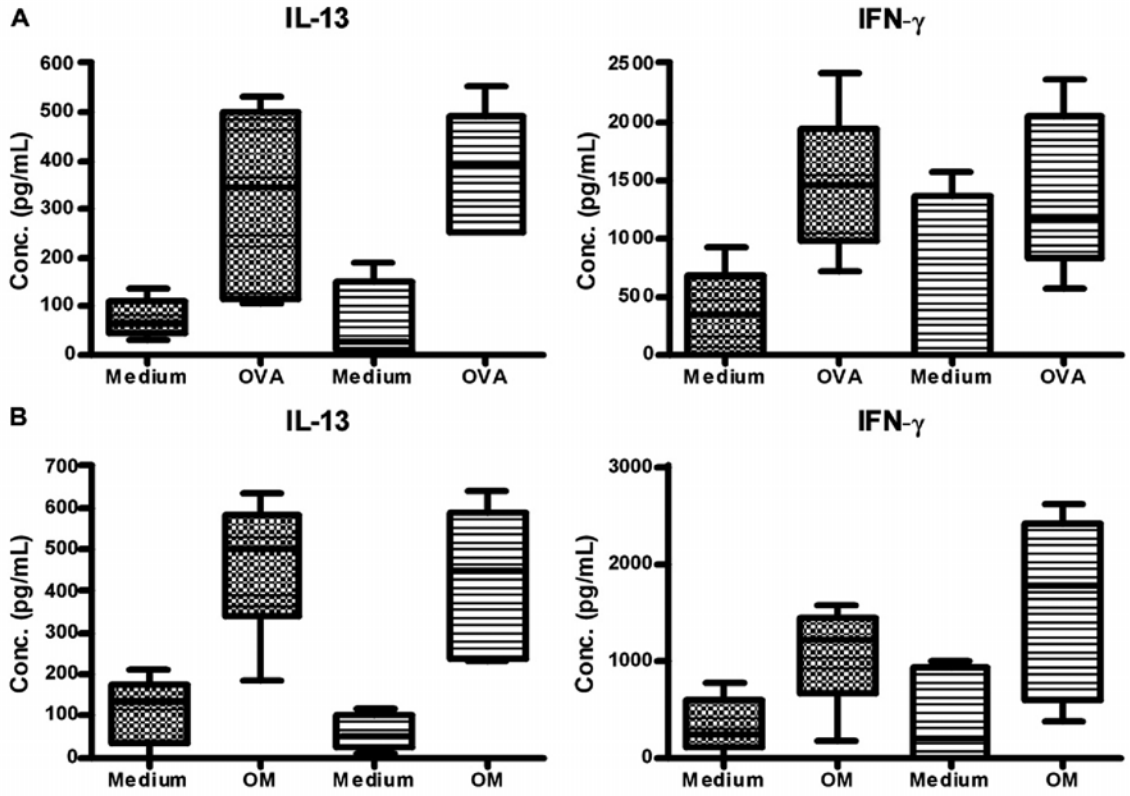

FIG E1. *In vitro* IL-13 and IFN-γ induction on stimulation with OVA or ovomucoid *(OM)* in cultured spleen cells from OVA-sensitized **(A)** or OM-sensitized **(B)** mice challenged with native *(dotted bars)* or heated *(striped bars)* OVA or OM. *Conc.,* Concentration.

J ALLERGY CLIN IMMUNOL
VOLUME 127, NUMBER 4

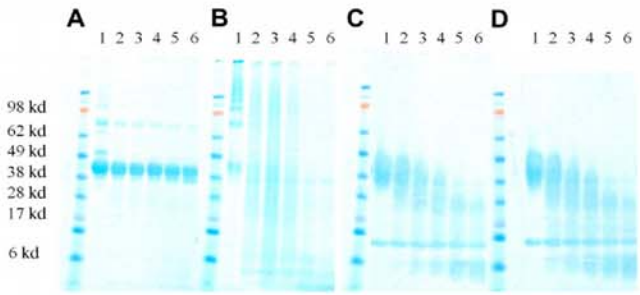

FIG E2. SDS-PAGE of unheated **(A)** and heated **(B)** OVA and of unheated **(C)** and heated **(D)** ovomucoid, subjected to pepsin digestion for 0, 1, 5, 10, 30, and 60 minutes (*lanes 1-6,* respectively).

VI

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Oral Immunotherapy induces local protective mechanisms in the gastrointestinal mucosa

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Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa

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ABSTRACT

Background: Oral immunotherapy (OIT) is a promising treatment for food allergy. Studies are needed to elucidate mechanisms of clinical protection, and to identify safer and potentially more efficacious methods for desensitizing patients to food allergens.

Objective: We established a mouse model of OIT in order to determine how dose or form of antigen may affect desensitization, and to identify mechanisms of desensitization.

Methods: Increasing doses of egg white or ovomucoid as OIT was administered orally to sensitized mice. Impact of OIT on anaphylaxis elicited by oral allergen challenge was determined. Allergen-specific antibody and cytokine responses, and mast cell and basophil activation in response to OIT was measured. Gene expression in the small intestine was studied by microarray and real-time PCR.

Results: OIT resulted in desensitization but not tolerance of mice to the allergen. OIT did not result in desensitization of systemic effector cells, and protection was localized to the gastrointestinal tract. OIT was associated with significant changes in gene expression by the intestinal epithelium. Extensively heated ovomucoid that does not trigger anaphylaxis when given orally to sensitized mice was as efficacious as native ovomucoid in desensitizing mice when administered as OIT.

Conclusions: OIT results in clinical protection against food-induced anaphylaxis through a novel mechanism that is localized to the intestinal mucosa and is associated with significant downregulation of epithelial-expressed genes. Extensively heating egg allergen decreases allergenicity and increases safety while still retaining the ability to induce effective desensitization.

Key Messages

- The mechanism of clinical protection induced by OIT is localized to the gastrointestinal tract and is associated with significant changes in epithelial gene expression.
- Extensively heated antigen that is unable to elicit anaphylaxis can effectively desensitize mice when used as OIT.

Capsule Summary

Heated egg protein may represent an alternative form of OIT that is safer, leading to fewer side effects and increasing adherence to therapy. Modifications of other allergens to suppress their uptake through the intestinal mucosa may also be an effective approach to generating safer allergens for use in OIT. Our findings point to intriguing gastrointestinal mechanisms that may underlie clinical responsiveness to food allergens.

Key Words

Oral immunotherapy

Food allergy

Heated egg allergen

Mucosal protection

Abbreviations

OIT – oral immunotherapy

SCIT – subcutaneous immunotherapy

OVA – ovalbumin

OM – ovomucoid

CT – cholera toxin

ip - intraperitoneal

INTRODUCTION

Food allergies affect 3% of the overall population and up to 4-6% of children.[1] From 1997 to 2007, the prevalence of reported food allergy increased 18% among children under 18 years of age.[2] Currently, there is no cure for food allergy and management remains avoidance of the offending food.[3] Interventions that would lessen the risk of anaphylaxis would have a major impact on the quality of life, morbidity, and mortality.

Subcutaneous immunotherapy (SCIT) has been used as treatment for other allergic diseases, such as allergic rhinitis, venom allergy and allergic asthma, but has not been successful in food allergy due to high rates of systemic reactions.[4, 5] There is an increasing interest in oral immunotherapy (OIT) for the treatment of food allergy, and several clinical trials have shown promising results.[6-10] Starting at doses unlikely to trigger reactivity, subjects ingest increasing amounts of allergen over the duration of months to years with most tolerating significantly more allergen by the end of the study compared to their baseline. Adherence suffers from the high prevalence of side effects (even though they may be mild) and clinical reactivity returns after discontinuation of OIT in a majority of treated subjects, demonstrating desensitization but not tolerance.[9, 11]

Extensive heating can render milk and egg allergens tolerable to the majority of milk- and egg-allergic patients. When tolerated, introducing extensively heated antigens into the diet of allergic individuals does not only improve quality of life but may also accelerate resolution of clinical reactivity to foods. Although placebo-controlled interventions have not been performed, inclusion of extensively heated egg or milk into the diet appears to be well-tolerated and the majority of subjects go on to develop tolerance to unheated egg or milk.[12-14]

Immunologic changes have been associated with desensitization in OIT clinical trials, including decreased basophil reactivity, decreased wheal size on skin prick test (signifying decreased mast cell reactivity), increased of serum antigen-specific IgG4 levels, and increased peripheral CD4+CD25+FOXP3+ regulatory T cells.[6, 8-10] Similar immune changes have been observed after inclusion of extensively heated milk into the diet, supporting the hypothesis that this may be functioning as an immunotherapy facilitating development of immune tolerance.[15, 16] However, it is not known to what extent these immunologic changes contribute to clinical protection, and this is difficult to test in human subjects.

OIT is a promising treatment and potential cure for food allergy, but issues of safety and long-term efficacy remain.[17] Mechanisms of clinical protection are difficult to investigate in human subjects, and the gastrointestinal mucosa cannot be practically accessed for study.

To address these needs, we established a murine model of OIT. We show that mice orally sensitized to egg allergens can be readily desensitized and protected from egg-induced anaphylaxis but not permanently tolerized, consistent with the typical human response to OIT. Using this mouse model of OIT, we show that extensively heated egg allergens that are non-reactogenic are as effective when used as OIT as native egg allergen, providing evidence for a safer alternative to current OIT protocols. Secondly, we show that the mechanism of clinical protection in OIT-treated mice resides in the gastrointestinal mucosa and is associated with significant changes in gene expression by a subset of intestinal epithelial cells.

METHODS

Mice: Female C3H/HeJ mice were purchased from the National Cancer Institute (Frederick, MD). Mice were maintained in filter-top cages under specific pathogen-free conditions. The Institutional Animal Care and Use Committee at Mount Sinai School of Medicine approved all procedures.

Sensitization of mice: Mice were sensitized by intragastric administration of antigen plus cholera toxin (CT) as adjuvant once a week for six weeks. Feedings consisted of 1 mg of ovalbumin (OVA, grade V, Sigma Chemicals, St. Louis, MO) or ovomucoid (OM, Sigma) plus 10 μg CT (List Biologicals, Campbell, CA, USA) in 0.4 ml of 0.2 M bicarbonate.

Administration of oral immunotherapy (OIT): Pilot experiments were done comparing the administration of antigen as OIT by daily gavage or through the drinking water, with the two methods being equally effective. Egg white was obtained from organic eggs as the source of egg protein and protein content measured by Bradford assay. Egg white rather than purified allergen was used as OIT primarily due to cost, but for sensitization and challenge the use of purified allergens provides more consistent sensitization and anaphylaxis responses. An increasing dose of egg protein was administered daily for 14 days: 1 mg (days 1,2), 5 mg (days 3,4), 10 mg (days 5-7), 25 mg (days 8,9), 50 mg (days 10-14). The dose was calculated based on a drinking volume of 5 ml/day. For experiments using extensively heated and native OM as OIT, mice received OM by daily gavage at half the dose of that used for whole egg white protein, (increasing doses from 0.5 mg to 25 mg). This dose of OM was equivalent to the dose of OVA provided in whole egg white. OM was heated as previously described.[18]

Allergen challenge: Mice were orally challenged on day 15 or two weeks after discontinuation of OIT with 50 mg OVA or 25 mg OM. For systemic challenges, mice were intraperitoneally injected with 100 μg of OVA in PBS. Anaphylaxis severity was graded by symptom score (Li, 1999) and body temperature was measured (by rectal thermometer, World Precision Instruments, Sarasota, FL) 30 minutes after challenge.

Assessment of antigen-specific immunoglobulins: Blood samples were obtained after sensitization before and after OIT. OVA- and OM-specific IgE was measured by a capture ELISA using DIG-labeled OVA or OM as detection.[19] OVA-specific IgA, IgG1, and IgG2a

were measured by ELISA using biotinylated monoclonal detection antibodies (all from BD Biosciences, San Diego, CA).

Antigen-specific cytokine production: Spleens were removed after challenge and cells isolated and plated with OVA or OM (100 μg/ml) for 72 h. Cytokine production was measured by ELISA (eBioscience, San Diego, CA).

Mast cell and basophil activation assays: For basophil activation, blood was collected from mice in heparinized tubes, diluted 1:1 with RPMI, and incubated at 37 ºC for 60 min with 10 – 100 μg/ml of OVA or media alone as control. Cells were then fixed, red blood cells lysed, and cells stained for CD49b and IgE to detect basophils, and CD200R as an activation marker.[20] CD3 and CD19 were included to gate out B and T cells. For mast cell activation, peritoneal lavage was collected and isolated cells were stimulated with 10 – 100 μg/ml of OVA or media alone as a control at 37 ºC for 60 min. Cells were stained for c-kit and IgE to detect mast cells, and CD107a (LAMP-1) as an activation marker.[21] All antibodies were from eBioscience (San Diego, CA).

Assessment of intestinal permeability: After discontinuing OIT one day prior, OIT-treated and control OVA-sensitized mice were euthanized, and 10 cm of jejunum were excised starting from the ligament of Treitz. Resistance and flux measurements were obtained as described previously.[22]

Intestinal gene expression: Intestine from OVA-sensitized mice, control and OIT-treated, was harvested 24 h after discontinuation of OIT. Two pieces of jejunum obtained 0 and 10 cm from the Ligament of Treitz and cleaned of mesentery were snap-frozen in Trizol (Invitrogen). Total RNA was isolated with Trizol followed by RNA clean-up with RNeasy mini-kit (Qiagen, Valencia, CA). Microarray analysis was performed by Miltenyi (Cologne, Germany) using Agilent whole mouse genome oligo microarrays. Paired analysis on 5 control and 5 OIT-treated tissues was performed using Cy3-labeled and Cy5-labeled cRNA. Gene expression was verified by real-time PCR using SYBR green master mix and primers (all from Invitrogen). Epithelial cells were isolated by EDTA treatment and RNA was isolated with an RNeasy mini-kit (Qiagen).

Statistical analysis: Student's t-test or Mann-Whitney Rank Sum test was used for determining statistical significance ($p < 0.05$) between continuous variables, and a paired t-test or Wilcoxon Signed Rank test was used when comparing different time points.

RESULTS

OIT reduces anaphylaxis by desensitization in a murine model of food allergy

To determine the effect of OIT in a murine model of food allergy, C3H/HeJ mice were orally sensitized to OVA or OM and then administered OIT as either fresh egg white or purified OM for two weeks. Mice were then orally challenged with OVA or OM. Administration of OIT was well tolerated. OIT-treated mice were significantly protected from OVA- or OM-induced anaphylaxis as compared to controls ($p<0.001$ for OVA, $p<0.01$ for OM; based on body temperature). **(Figure 1)** Symptom scores were also significantly reduced. After discontinuing OIT for two weeks, mice were re-challenged to assess tolerance. OIT-treated mice were no longer protected from anaphylaxis, indicating that OIT induced clinical protection through desensitization, not immune tolerance.

OIT induces an increase in OVA-specific serum IgE and IgA

OVA-specific serum immunoglobulins were measured after OIT treatment prior to challenge. A significant increase in OVA-specific IgE and IgA was observed in OVA OIT-treated mice as compared to controls. **(Figure 2a)** Serum OVA-specific IgG1 and IgG2a, which have been shown to inhibit IgE-triggered mast cell activation [23], were not significantly different between OIT-treated mice and controls. Measurements of OM-specific serum immunoglobulins in OM OIT-treated mice were similar (data not shown). OVA-specific IgA in gut lavage could be detected and was significantly elevated in OVA-sensitized mice compared to naïve mice, but was not significantly increased by the administration of OIT (data not shown).

OIT is associated with a broad suppression of cytokines

The impact of OIT on antigen-specific T cell responses was assessed by re-stimulating splenocytes with OVA or OM *in vitro* and measuring cytokine secretion. Antigen-specific IL-13, IL-10, and IFN-γ responses were all significantly reduced in OIT-treated mice as compared to controls, indicating broad suppression rather than skewing of T cell responses. **(Figure 2b)** When OIT was discontinued for two weeks, a sustained suppression of cytokines was observed in OIT-treated mice as compared to controls despite the return of clinical responsiveness.

OIT results in decreased intestinal barrier function

Gastrointestinal side effects have been identified as a major early obstacle to OIT compliance.[24] In order to determine the impact of OIT on intestinal physiology, segments of jejunum were collected from OIT-treated mice as well as controls and mounted in Ussing chambers. Epithelial barrier function was measured by resistance as well as luminal-to-serosal flux of FITC-dextran. **(Figure 3)** OIT resulted in a significant decrease in resistance, and significant increase in luminal to serosal flux of FITC-dextran, indicating a decrease in epithelial barrier function.

High-dose OIT is required for clinical protection

Low doses of antigen preferentially elicit regulatory T cells that can prevent systemic immune responses in classic oral tolerance studies.[25] To determine if low-dose OIT could be effective, OVA-sensitized mice were administered either a daily low dose of 1 mg (the dose typically used in low-dose tolerance studies[26]) or escalating daily doses of up to 50 mg (high dose, or the standard OIT used in this model) for two weeks and then orally challenged with 50 mg of OVA. Upon oral challenge, high-dose OIT-treated mice were protected against anaphylaxis ($p<0.001$), while low-dose OIT-treated mice were not. **(Figure 4)** Despite the lack of clinical protection, OVA-stimulated spleen cells from both high- and low-dose OIT-treated mice showed similar *in vitro* cytokine suppression compared to controls. (data not shown)

Extensively heated antigen effectively desensitizes mice

We have previously shown that heating of OVA or OM abolishes their capacity to trigger anaphylaxis when given orally, but not systemically, to sensitized mice.[18] We investigated whether this non-reactogenic form of antigen could be effective when administered as OIT. OM was chosen because OVA coagulates after heating and is more difficult to administer by gavage than heated OM. Mice administered either heated or native OM as OIT were completely protected against anaphylaxis as compared to controls. **(Figure 5)** These data demonstrate that allergens can be modified to reduce risks of systemic anaphylaxis, yet still maintain their full desensitizing capacity.

Protective mechanisms induced by OIT are localized to the gastrointestinal tract

To test if OIT leads to desensitization of systemic effector cells, we performed activation assays with peripheral blood basophils and peritoneal mast cells. Heparinized

blood from OIT-treated or control mice was incubated with OVA *in vitro,* followed by assessment of basophil activation by flow cytometry. Basophils were detected as $CD49b^+$ IgE^+ cells, and activation measured by up-regulation of CD200R. Blood basophils from control OVA-sensitized mice upregulated CD200R in response to OVA stimulation, and this was not suppressed in OIT-treated OVA-sensitized mice. **(Figure 6a)** Activation of peritoneal mast cells (c-kit^+ $Fc\varepsilon RI^+$) in response to OVA stimulation *in vitro* was measured by upregulation of the marker LAMP-1, or CD107a. Similar to basophil activation, mast cell activation was not suppressed in mice administered OIT. (**Figure 6b**)

We hypothesized that clinical protection against food-induced anaphylaxis may be occurring locally within the intestinal mucosa. To test this, OVA-sensitized mice were challenged by either the oral route or systemically by low-dose intraperitoneal injection. OIT-treated mice were protected against anaphylaxis induced by oral challenge, but were not protected against anaphylaxis induced by systemic challenge. (**Figure 6c**)

We next tested the antigen specificity of the protective effect of OIT. Mice were simultaneously sensitized to both OVA and OM. Mice then underwent OIT with OM, followed by oral challenge with OVA to assess bystander desensitization. Mice administered OM-OIT were significantly protected from anaphylaxis induced by OVA (**Figure 6d**).

OIT induces significant changes in epithelial gene expression

To begin to understand the impact of OIT locally within the gastrointestinal tract, we performed gene expression microarray analysis on jejunum from sensitized control mice compared to those who had undergone OIT. A list of genes regulated (positively or negatively) at least two-fold was compiled. Genes regulated in at least 4 of 5 control:OIT comparisons were identified. No genes were observed to be consistently up-regulated, but 23 genes were found to be significantly down-regulated in a minimum of 4 of 5 comparisons (Table 1). These could be broadly grouped into two categories: digestive enzymes such as trypsin, carboxypeptidase, lipase, and amylase; and antimicrobial peptides such as alpha-defensins. Down-regulation of representative genes was confirmed by real-time PCR (Figure 7). Several of these genes, such as alpha-defensins and trypsins, have been reported to be expressed primarily by paneth cells of the small intestine. To confirm small intestinal expression of these target genes, expression of two representative genes, pancreatic lipase (pnlp) and trypsin 4 (try4) was examined in segments from proximal to distal small intestine, carefully cleaned of mesentery. Gene expression was maximal in the duodenum, and

decreased from jejunum to ileum. Intestinal crypt epithelium was isolated by EDTA treatment, and expression of pnlp and try4 compared between intact intestine and isolated epithelium. Expression was enriched > 60-fold for pnlp, and > 90-fold for try4 in isolated epithelial cells as compared to whole gut. These data show that epithelial-expressed genes of the small intestine are significantly altered by the administration of OIT.

DISCUSSION

OIT is a promising therapy for food allergy with mechanisms of clinical protection that are not well understood. We have developed a murine model of OIT for the treatment of food-induced anaphylaxis that induces clinical protection and is dependent on continued exposure to allergen. This response in sensitized mice closely resembles the typical human response to OIT. Using this model, we have generated two main novel findings. The first is that the mechanism of clinical protection is localized to the gastrointestinal tract and is associated with significant changes in epithelial gene expression. The second is that extensively heated antigen that is unable to elicit anaphylaxis can effectively desensitize mice.

Immunologic studies from human OIT trials have shown variable effects on antigen-specific IgE and consistent increases in antigen-specific IgG4. Our data indicates that clinical reactivity to food allergens can be abrogated without reducing allergen-specific IgE or increasing allergen-specific IgG. IgA is one potential mechanism of protection. We observed a significant increase in serum allergen-specific IgA associated with OIT, but no change in gut lavage allergen-specific IgA. Strait et al recently reported that IgA could suppress anaphylaxis by neutralizing absorbed antigen in the circulation rather than by preventing uptake of antigen.[27] Arguing against a significant role for serum IgA in our studies is the finding that protection was only observed in response to oral allergen challenge, not low-dose systemic allergen challenge, while changes in IgA were found systemically but not at the gut level. Secondly, we observed that OIT could result in significant protection against a bystander antigen, suggesting that the mechanism of protection may not be based on specific antigen recognition. A bystander effect has also been reported in a model of tree nut immunotherapy.[28]

Hypo-responsiveness of allergic effector cells, as measured by skin-prick test or *in vitro* basophil activation, has been variably reported in human OIT trials. It is theorized that mast cells and basophils are degranulated gradually by immunotherapy until they become unresponsive or are in a refractory state.[29] Skin-prick tests have been observed to be unchanged compared to placebo [8] or decreased [9, 10], and *in vitro* basophil activation tests have been shown to be unchanged [30] or decreased [31]. Our current results showed that administration of OIT did not result in any changes in responsiveness of peripheral blood basophils or peritoneal mast cells to activation. Basophil activation assays were performed in whole blood and would therefore reflect the antibody milieu. Mast cells have been shown to

be the primary cells responsible for systemic anaphylaxis in mice after sensitization with cholera toxin adjuvant[32, 33], with basophils playing a minor role [33]. This suggests that the mechanism of protection induced by OIT is upstream of systemic mast cell activation. Our results are consistent with those reported by Skripak et al, where significant clinical protection was observed despite a lack of significant effect of OIT on tissue mast cells compared to placebo.[8] The finding that clinical protection can be observed in the absence of changes in mast cell or basophil activation suggests that there may be alternative mechanisms at play, possibly involving local mechanisms in the gastrointestinal mucosa as we are reporting.

We observed that clinical protection in response to OIT was localized to the gastrointestinal tract, which suggests a novel mechanism of desensitization that may be unique to food allergy. Anaphylaxis in response to systemic allergen challenge was unaffected by OIT. Although high dose systemic challenge can elicit IgG-mediated mechanisms of anaphylaxis[33], we used 0.2% of the dose given orally for systemic challenge. This is in the range of what we would expect to be absorbed intact into the circulation from the gastrointestinal tract after allergen challenge. Furthermore, we observed no evidence of systemic effector cell desensitization. This suggests that there is a mechanism of allergen detection or uptake from the gut that is blocked by OIT. Microarray studies showed that OIT was associated with a significant downregulation of gene expression in the proximal jejunum. Genes could be divided into two main categories: digestive enzymes such as proteases, lipases, and amylases; and anti-microbial peptides (α-defensin-related peptides). The digestive enzymes were surprising as the main source of these enzymes is the pancreas. The down-regulation of two representative enzymes, pancreatic lipase (pnlp) and trypsin 4 (try4) was verified by real-time PCR in the intestine, and expression was confirmed in the duodenum and proximal jejunum and found to be expressed primarily by the epithelium. A number of pancreatic enzymes, including trypsin, are also expressed by intestinal Paneth cells that are an important source of anti-microbial peptides including α-defensins. The function of these enzymes in the Paneth cell remains unknown, although some enzymes are involved in proteolytic processing of anti-microbial peptides.[34] Our data indicates that a subset of intestinal epithelial cells is significantly modified by the administration of OIT. The link between these changes in epithelial gene expression and clinical protection needs to be determined, as does the applicability of these findings to the human response to OIT. These

findings point to intriguing gastrointestinal mechanisms that may underlie clinical responsiveness to food allergens.

Persistent side effects are a roadblock to the adoption of OIT as a widespread therapy for food allergy. These include both systemic and gastrointestinal side-effects, and consistent with the latter we have observed that OIT is associated with significant decrease in epithelial barrier function. This is likely due to local mast cell activation that is known to modulate the epithelial barrier.[35] Clinical studies have shown that a majority of egg- and milk-allergic individuals can tolerate baked egg or milk and it is anticipated that incorporating heated forms of allergen into their diet will accelerate resolution of their allergy.[12-14] Furthermore, assessment of intestinal permeability in these subjects has shown no negative effects of incorporation of extensively heated milk into the diet on intestinal barrier function.[12] We have previously shown that heated egg allergens cannot elicit anaphylaxis in sensitized mice due to changes in uptake of intact antigen across the intestinal mucosa.[18] Similarly, we have found that extensively heated milk allergens cannot elicit anaphylaxis when given orally due to altered trafficking across intestinal epithelial cells.[36] In this study, we found that heated egg allergen can nevertheless be used as OIT, and is as effective as native antigen in suppressing anaphylaxis. Our results with heated egg allergens can be directly translated into an alternative form of OIT that is safer, leading to fewer side effects and increasing adherence to therapy in clinical trials. Alternatively, extensively heated allergens could be used as a lead-in to desensitize patients prior to OIT with native forms of allergen. Modifications of allergens other than egg or milk to suppress their uptake through the intestinal mucosa may also be an effective approach to generating safer allergens for use in OIT.

FIGURE LEGENDS

Figure 1. Oral immunotherapy (OIT) results in desensitization but not tolerance to OVA or OM. OVA- and OM-sensitized mice were administered egg white or OM OIT, or left untreated (Control). Mice were orally challenged on day 15 (*on OIT*), and again two weeks after discontinuation of OIT (*off OIT*). 30 minutes after oral challenge with 50 mg of OVA or 25 mg OM, body temperature was measured (left) and a clinical score was assigned (right).

Figure 2. Immunologic changes associated with OIT in mice. Mice were orally sensitized to OVA, followed by administration of egg white OIT. (A) Blood was obtained on the last day of OIT, and OVA-specific immunoglobulins were measured by ELISA. (B) Spleen cells were cultured and re-stimulated *in vitro* with 100 μg/ml of OVA. Cytokines were measured by ELISA in culture supernatants harvested after 72 h. * $p < 0.05$

Figure 3: OIT decreases gastrointestinal epithelial barrier function. Mice were orally sensitized to OVA, followed by administration of egg white OIT. On day 15, segments of jejunum were removed and mounted in Ussing chambers. Transmural resistance was measured after a 30-minute equilibration period (left), and luminal to serosal transport of FITC-dextran was measured over a 90-minute period (right). ** $p < 0.01$.

Figure 4: High dose OIT is required for clinical protection against OVA-induced anaphylaxis in sensitized mice. OVA-sensitized mice were administered high-dose (increasing up to 50 mg/day) or low-dose (1 mg/day) fresh egg white OIT, or left untreated (Control). Mice were orally challenged with 50 mg of OVA on day 15. 30 minutes after challenge, body temperature was measured (left) and a clinical score was assigned (right).

Figure 5: Extensively-heated OM protects mice against anaphylaxis. OM-sensitized mice were administered native or heated-OM OIT, or left untreated (Control). Mice were challenged with 25 mg of native OM on day 15. 30 minutes after oral challenge, body temperature was measured (left) and a clinical score was assigned (right).

Figure 6: Clinical protection is localized to the gastrointestinal tract. Mice were orally sensitized to OVA, followed by egg white OIT. (A) Peripheral blood from control or OIT-

treated mice was stimulated with media or OVA, followed by flow cytometry for the activation marker CD200R. One representative experiment of three is shown. (B) Peritoneal cells were harvested and stimulated with media or OVA for 60 min. LAMP-1 on mast cells was used as an activation marker and quantified as median fluorescence intensity (MFI). Data are mean + SEM from 5 mice/group. (C) Control or OIT-treated OVA-sensitized mice were challenged with 50 mg OVA orally or 0.1 mg OVA ip. Body temperature was measured pre- and post-challenge. (D) Mice were orally sensitized to OVA plus OM, followed by OIT with OM. Mice were challenged with OVA, and body temperature measured after challenge.

Figure 7: OIT significantly alters intestinal epithelial gene expression. (A) Mice were orally sensitized to OVA, followed by administration of egg white OIT. RNA was isolated from jejunum, and expression of pancreatic lipase (pnlp), trypsin 4 (try4), trypsin 5 (try5) and GP2 was measured by real-time PCR. (B) RNA was isolated from the small intestine (proximal and mid-duodenum, jejunum, and proximal and distal ileum) and expression of pnlp and try4 was measured (left). From a segment of duodenum, epithelial cells were isolated and expression of pnlp and try4 were measured in isolated intestinal epithelial cells (IEC) compared to whole duodenum (right).

Table 1: List of genes regulated by OIT in 4 of 5 comparisons

Gene	Fold Change	SEM
Amylase	-124.8	53.9
VH mRNA	-3.0	0.6
Amylase 1	-45.8	14.2
Amylase 2a5	-50.9	15.3
Carboxyl Ester Lipase	-11.1	4.2
Carboxypeptidase A1	-19.8	1.2
Carboxypeptidase A2	-27.1	7.9
Carboxypeptidase B2	-143.9	66.2
Chymotrypsin C	-21.5	8.0
Elastase 2A	-31.1	9.5
Elastase 3B	-29.9	5.4
Chymotrypsinogen B1	-8.2	1.1
α-defensin, RS12	-5.5	1.5
α-defensin, RS2	-7.8	2.0
α-defensin, RS4	-5.2	1.1
GP2	-10.1	2.6
Pancreatic Lipase	-111.1	11.4
Phospholipase A2	-12.7	3.4
Protein disulfide isomerase a2	-12.3	3.8
Syncollin	-18.3	8.6
Trypsin 4	-114.5	15.8
Trypsin 5	-173.1	14.5
Trypsin 1	-38.8	7.8

REFERENCES

1. Sicherer S. Epidemiology of food allergy. J Allergy Clin Immunol 2011.
2. Branum AM, Lukacs SL. Food allergy among U.S. children: trends in prevalence and hospitalizations. NCHS Data Brief 2008:1-8.
3. Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the Diagnosis and Management of Food Allergy in the United States: Summary of the NIAID-Sponsored Expert Panel Report. J Allergy Clin Immunol; 126:1105-18.
4. Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. J Allergy Clin Immunol 2009; 123:735-46; quiz 47-8.
5. Nelson HS, Lahr J, Rule R, Bock A, Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. J Allergy Clin Immunol 1997; 99:744-51.
6. Buchanan AD, Green TD, Jones SM, Scurlock AM, Christie L, Althage KA, et al. Egg oral immunotherapy in nonanaphylactic children with egg allergy. J Allergy Clin Immunol 2007; 119:199-205.
7. Staden U, Rolinck-Werninghaus C, Brewe F, Wahn U, Niggemann B, Beyer K. Specific oral tolerance induction in food allergy in children: efficacy and clinical patterns of reaction. Allergy 2007; 62:1261-9.
8. Skripak JM, Nash SD, Rowley H, Brereton NH, Oh S, Hamilton RG, et al. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. J Allergy Clin Immunol 2008; 122:1154-60.
9. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschorner J, de Oliveira LC, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. J Allergy Clin Immunol 2010; 126:83-91 e1.
10. Varshney P, Jones SM, Scurlock AM, Perry TT, Kemper A, Steele P, et al. A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. J Allergy Clin Immunol 2011; 127:654-60.
11. Rolinck-Werninghaus C, Staden U, Mehl A, Hamelmann E, Beyer K, Niggemann B. Specific oral tolerance induction with food in children: transient or persistent effect on food allergy? Allergy 2005; 60:1320-2.

12. Lemon-Mule H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, Nowak-Wegrzyn A. Immunologic changes in children with egg allergy ingesting extensively heated egg. J Allergy Clin Immunol 2008; 122:977-83 e1.
13. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, et al. Tolerance to extensively heated milk in children with cow's milk allergy. J Allergy Clin Immunol 2008; 122:342-7, 7 e1-2.
14. Kim JS, Nowak-Wegrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA. Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol 2011; 128:125-31 e2.
15. Shreffler WG, Wanich N, Moloney M, Nowak-Wegrzyn A, Sampson HA. Association of allergen-specific regulatory T cells with the onset of clinical tolerance to milk protein. J Allergy Clin Immunol 2009; 123:43-52 e7.
16. Wanich N, Nowak-Wegrzyn A, Sampson HA, Shreffler WG. Allergen-specific basophil suppression associated with clinical tolerance in patients with milk allergy. J Allergy Clin Immunol 2009; 123:789-94 e20.
17. Thyagarajan A, Varshney P, Jones SM, Sicherer S, Wood R, Vickery BP, et al. Peanut oral immunotherapy is not ready for clinical use. J Allergy Clin Immunol 2010; 126:31-2.
18. Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC, Nowak-Wegrzyn A. Mechanisms underlying differential food allergy response to heated egg. J Allergy Clin Immunol 2011; 127:990-7 e1-2.
19. Blázquez A, Mayer… L. Thymic Stromal Lymphopoietin is Required for Gastrointestinal Allergy but not Oral Tolerance. Gastroenterology 2010.
20. Torrero MN, Larson D, Hubner MP, Mitre E. CD200R surface expression as a marker of murine basophil activation. Clin Exp Allergy 2009; 39:361-9.
21. Grutzkau A, Smorodchenko A, Lippert U, Kirchhof L, Artuc M, Henz BM. LAMP-1 and LAMP-2, but not LAMP-3, are reliable markers for activation-induced secretion of human mast cells. Cytometry A 2004; 61:62-8.
22. Knight AK, Blázquez AB, Zhang S, Mayer L, Sampson HA, Berin MC. CD4 T cells activated in the mesenteric lymph node mediate gastrointestinal food allergy in mice. Am J Physiol Gastrointest Liver Physiol 2007; 293:G1234-43.
23. Uermosi C, Beerli RR, Bauer M, Manolova V, Dietmeier K, Buser RB, et al. Mechanisms of allergen-specific desensitization. J Allergy Clin Immunol 2010; 126:375-83.

24. Vickery BP, Scurlock AM, Steele P, Kamilaris J, Hiegel AM, Carlisle SK, et al. Early and persistent gastrointestinal side effects predict withdrawal from peanut oral immunotherapy (OIT). J Allergy Clin Immunol 2011; 127:AB26.
25. Chen YH, Weiner HL. Dose-dependent activation and deletion of antigen-specific T cells following oral tolerance. Ann N Y Acad Sci 1996; 778:111-21.
26. Kraus TA, Brimnes J, Muong C, Liu JH, Moran TM, Tappenden KA, et al. Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops. J Clin Invest 2005; 115:2234-43.
27. Strait RT, Mahler A, Hogan S, Khodoun M, Shibuya A, Finkelman FD. Ingested allergens must be absorbed systemically to induce systemic anaphylaxis. J Allergy Clin Immunol 2011; 127:982-9 e1.
28. Kulis M, Li Y, Lane H, Pons L, Burks W. Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts. J Allergy Clin Immunol 2011; 127:81-8.
29. Castells M. Rapid desensitization for hypersensitivity reactions to medications. Immunol Allergy Clin North Am 2009; 29:585-606.
30. Frischmeyer-Guerrerio PA, Chichester K, Bieneman A, Keet C, Wood RA, Schroeder JT. Basophil Responses in Children Undergoing Immunotherapy for Milk Allergy. J Allergy Clin Immunol 2011; 127:AB26.
31. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, et al. Clinical efficacy and immune regulation with peanut oral immunotherapy. The J Allergy Clin Immunol 2009; 124:292-300, .e1-97.
32. Sun J, Arias K, Alvarez D, Fattouh R, Walker T, Goncharova S, et al. Impact of CD40 ligand, B cells, and mast cells in peanut-induced anaphylactic responses. J Immunol 2007; 179:6696-703.
33. Arias K, Chu DK, Flader K, Botelho F, Walker T, Arias N, et al. Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. J Allergy Clin Immunol 2011; 127:1552-61 e1.
34. Ghosh D, Porter E, Shen B, Lee SK, Wilk D, Drazba J, et al. Paneth cell trypsin is the processing enzyme for human defensin-5. Nat Immunol 2002; 3:583-90.
35. Groschwitz KR, Ahrens R, Osterfeld H, Gurish MF, Han X, Abrink M, et al. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. Proc Natl Acad Sci U S A 2009; 106:22381-6.

36. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy 2008; 63:882-90.

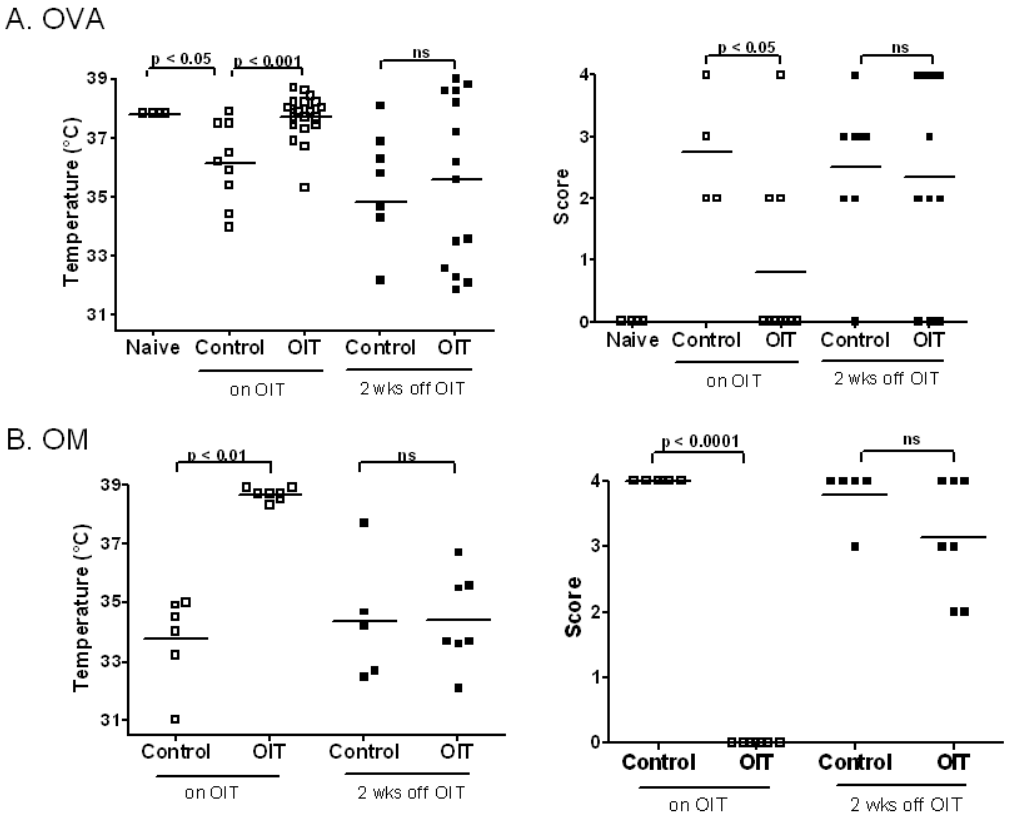
A. OVA
p < 0.05
p < 0.001
ns
Temperature (°C)
39
37
35
33
31
Naive
Control
OIT
Control
OIT
on OIT
2 wks off OIT
p < 0.05
ns
Score
4
3
2
1
0
Naive
Control
OIT
Control
OIT
on OIT
2 wks off OIT
B. OM
p < 0.01
ns
Temperature (°C)
39
37
35
33
31
Control
OIT
Control
OIT
on OIT
2 wks off OIT
p < 0.0001
ns
Score
4
3
2
1
0
Control
OIT
Control
OIT
on OIT
2 wks off OIT

Figure 1

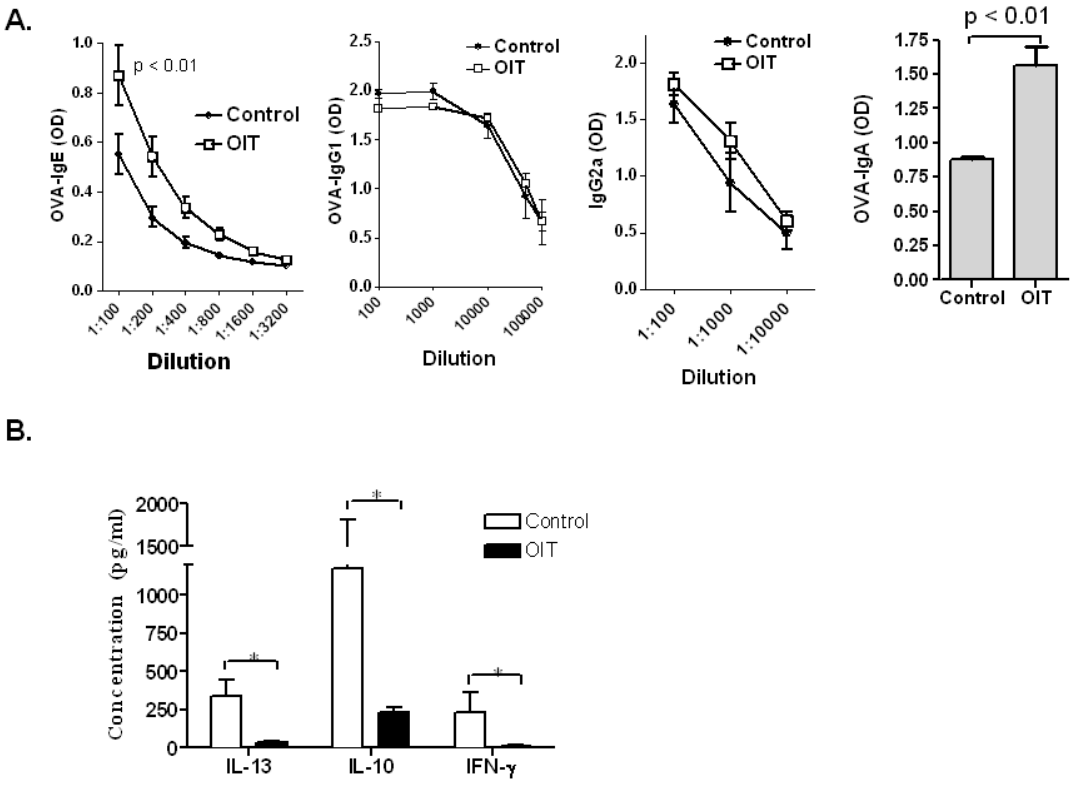
A.
p < 0.01
Control
OIT
OVA-IgE (OD)
1:100
1:200
1:400
1:800
1:1600
1:3200
Dilution
OVA-IgG1 (OD)
100
1000
10000
100000
Dilution
IgG2a (OD)
1:100
1:1000
1:10000
Dilution
p < 0.01
OVA-IgA (OD)
Control
OIT
B.
Concentration (pg/ml)
Control
OIT
IL-13
IL-10
IFN-γ

Figure 2

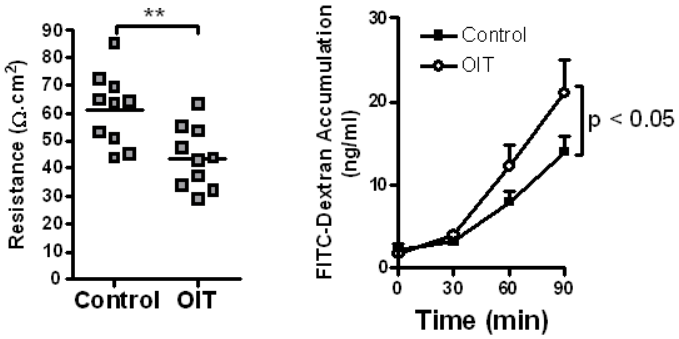
**
Resistance (Ω.cm²)
90
80
70
60
50
40
30
20
10
0
Control
OIT
FITC-Dextran Accumulation (ng/ml)
30
20
10
0
Control
OIT
p < 0.05
0
30
60
90
Time (min)

Figure 3

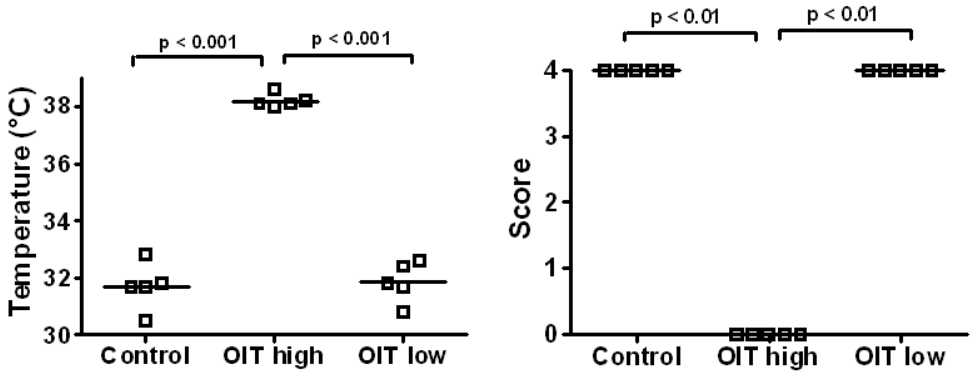
p < 0.001
p < 0.001
Temperature (°C)
38
36
34
32
30
Control
OIT high
OIT low
p < 0.01
p < 0.01
Score
4
3
2
1
0
Control
OIT high
OIT low

Figure 4

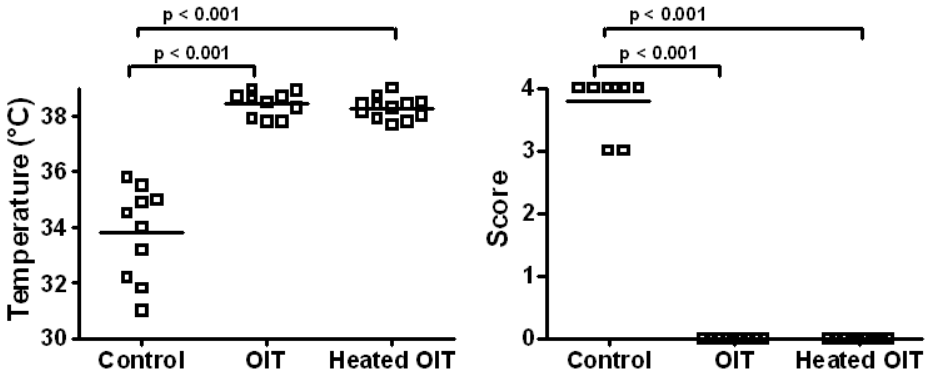
p < 0.001
p < 0.001
Temperature (°C)
38
36
34
32
30
Control
OIT
Heated OIT
p < 0.001
p < 0.001
Score
4
3
2
1
0
Control
OIT
Heated OIT

Figure 5

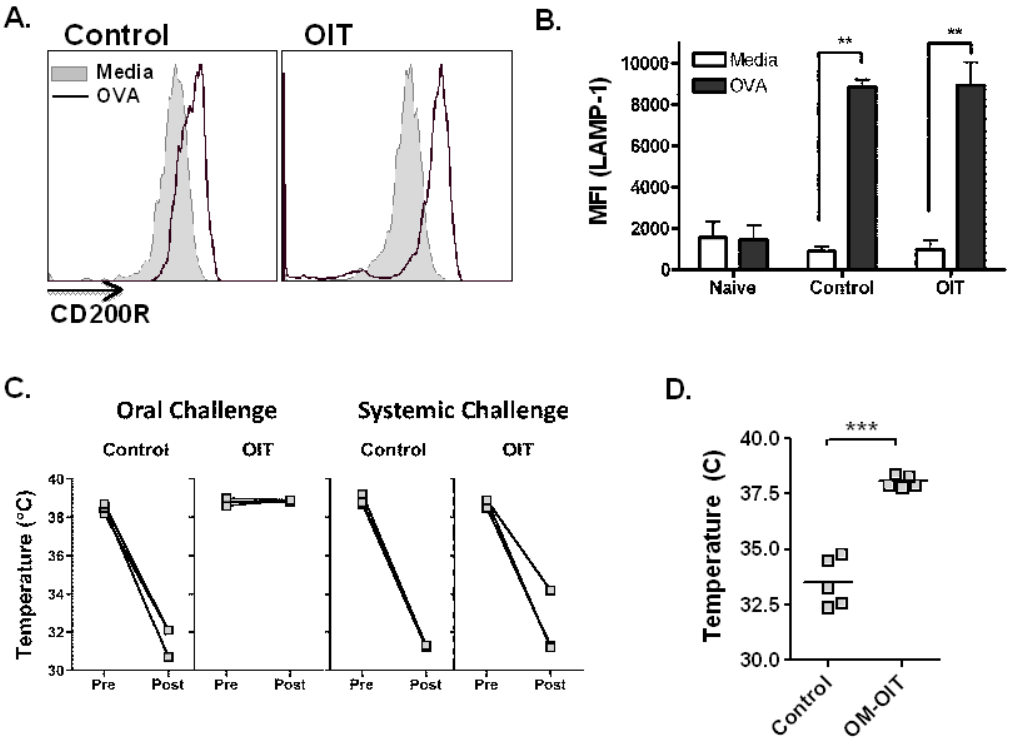
A.
Control
OIT
Media
OVA
CD200R
B.
MFI (LAMP-1)
Media
OVA
**
**
Naive
Control
OIT
C.
Oral Challenge
Systemic Challenge
Control
OIT
Control
OIT
Temperature (°C)
Pre
Post
D.
***
Temperature (C)
Control
OM-OIT

Figure 6

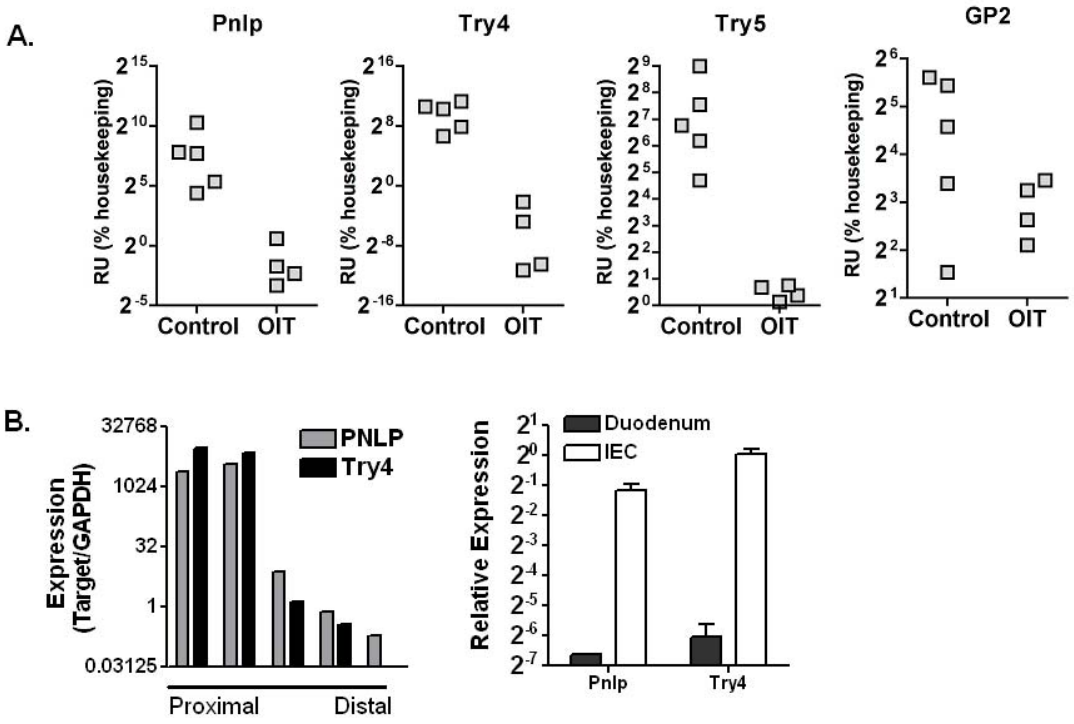
A.
PnIp
Try4
Try5
GP2
RU (% housekeeping)
Control
OIT
B.
PNLP
Try4
Expression (Target/GAPDH)
32768
1024
32
1
0.03125
Proximal
Distal
Relative Expression
Duodenum
IEC
PnIp
Try4

Figure 7

3. DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

Numerosos estudios apuntan hacia un aumento de las alergias alimentarias en las últimas décadas.[5, 124-126] El conocimiento actual acerca de las propiedades de las proteínas alergénicas pone de manifiesto la necesidad de profundizar en las características que convierten a un alimento en alergénico y en la comprensión de los mecanismos bioquímicos responsables de la alergia alimentaria, así como de desarrollar la investigación traslacional, del laboratorio a la clínica, que posibilite el progreso de las terapias encaminadas a restablecer la situación normal de tolerancia al alimento ofensivo. En la presente tesis, se aborda el estudio del comportamiento de las proteínas del huevo durante la digestión gastrointestinal en diferentes condiciones, con objeto de contribuir a explicar su alergenicidad. Asimismo, se estima la influencia de algunos factores que influyen en su digestibilidad e inmunogenicidad, como el tratamiento térmico o la concurrencia de otros componentes de la matriz alimentaria, que pueden proporcionar datos adicionales sobre el comportamiento de las proteínas alergénicas.

Al combinar información sobre las características fisicoquímicas intrínsecas al alérgeno con aquella obtenida de estudios sobre su interacción con el sistema inmunitario nos aproximamos en mayor medida al fenómeno alérgico. Por ello, en este trabajo también se han llevado a cabo estudios *in vivo* con ovoproteínas tratadas térmicamente para elucidar los mecanismos que originan el desencadenamiento de síntomas clínicos o la desensibilización mediante inmunoterapia oral. Conjuntamente con ensayos *in vitro* de absorción intestinal, activación de células efectoras y expresión génica, se ha logrado aportar datos que contribuyen al conocimiento actual de las alergias alimentarias.

A continuación, se discutirán los resultados obtenidos divididos en tres bloques: la digestibilidad de algunos alérgenos del huevo y su influencia en la reactividad frente a IgE, la interpretación bioquímica de la reducida alergenicidad de los alérgenos calentados, y el mecanismo de la desensibilización mediante inmunoterapia oral.

3.1 Digestibilidad de alérgenos del huevo y su influencia en la reactividad frente a IgE

La digestión fisiológica es un proceso vital y necesario, no solo para la correcta asimilación de los nutrientes de la dieta, sino también para que los componentes del alimento sean reconocidos adecuadamente por el sistema inmunitario intestinal sin provocar reacciones adversas. Para inducir una respuesta mediada por IgE, los alérgenos deben atravesar la barrera intestinal manteniendo un cierto grado de integridad estructural. Por ello, la resistencia a la digestión gastrointestinal se considera una característica específica de los alergenos alimentarios.[103] Sin embargo, también se han identificado varias proteínas alergénicas que son sensibles a la proteolisis.[100] Esto subraya la importancia de tener en cuenta todos los factores que pueden influir fisiológicamente en la digestión y de evaluar la capacidad de los fragmentos generados de interaccionar con IgE, a la hora de valorar su influencia en la alergenicidad de las proteínas alimentarias. Por otra parte, cuando el complejo proceso digestivo se ve alterado en alguna de sus etapas, las señales que recibe el sistema inmunitario pueden ser inadecuadas y conducir a un desequilibrio. Diversos estudios evidencian que la digestión incompleta o dificultada de ciertas proteínas alimentarias aumenta el riesgo de sensibilización o de reacción anafiláctica.[50, 127, 128] Nuestro objetivo ha sido establecer cómo la digestión de los alérgenos del huevo, en distintas condiciones, repercute en su capacidad final para ser reconocidos por la IgE del suero de pacientes alérgicos. La facultad de las proteínas digeridas para reaccionar con los anticuerpos IgE específicos las haría potencialmente capaces de inducir la desgranulación de mastocitos y basófilos y, en consecuencia, de producir los síntomas clínicos de la alergia.

En las primeras etapas de la digestión, el alimento llega al estómago, donde impera un pH ácido. Los ensayos de digestibilidad realizados han revelado que la hidrólisis enzimática por pepsina de las proteínas ovalbúmina (OVA) y lisozima (LYS) depende del pH (*resultados 2.1 y 2.2*). La digestión de OVA es limitada pero se favorece a pHs bajos, pH 1.2 y 2, siendo este efecto especialmente notorio al emplear pepsina en concentraciones próximas a las que se consideran fisiológicas (enzima/sustrato 1:20, p:p). Aunque la concentración gástrica de pepsina esta sujeta a una alta variabilidad tanto intra- como inter-individuo, parece claro que la cantidad de proteína superaría a la de enzima en la mayoría

de las circunstancias *in vivo*.[129] Esta influencia del pH ha sido ignorada en muchos estudios que, además, han evaluado la digestibilidad de OVA usando relaciones pepsina/sustrato muy superiores, tales como 19:1,[1] 13: 1[100] u 8:1[130] y cuyos resultados habrían subestimado la cantidad de proteína que llega intacta al duodeno. Por otra parte, diversos estudios han evaluado la contribución de la digestión de la LYS a la formación de péptidos con función defensiva en el intestino.[131] No obstante, el papel de la digestión en la producción de péptidos potencialmente capaces de reaccionar con la IgE de individuos alérgicos al huevo no ha sido investigado. Hemos constatado que sólo una elevada acidez (pH 1.2) resulta en la degradación completa del alérgeno por pepsina, mientras que a pH 2 una porción importante de proteína permanece sin digerir.

Dado que la pepsina presenta una actividad óptima en un amplio rango de pH (de 1.2 a 3.5), nuestros resultados respecto a OVA y LYS ponen de manifiesto la importancia de la acidez en la estructura y susceptibilidad enzimática de estos alérgenos. La OVA adopta a pH ácido una conformación de "molten globule", caracterizada por la pérdida parcial de su estructura terciaria y por una mayor flexibilidad, tal y como evidencian los datos de dicroísmo en el UV cercano. No obstante, retiene un grado suficiente de integridad estructural como para resistir la pepsinolisis en gran medida. La LYS, por su parte, adopta una conformación intermedia, parcialmente desnaturalizada, a pHs muy bajos (pH 1.5),[94] lo que podría ser la causa de la mayor susceptibilidad enzimática observada a pH 1.2, mientras que su conformación a pH 2 es igual que a pH neutro, lo que explicaría su mayor resistencia.

El valor del pH gástrico puede variar sustancialmente dependiendo de la edad del individuo,[132] condiciones pre- o posprandiales,[133] los alimentos ingeridos,[105] o factores extrínsecos como el consumo de medicamentos anti-acidez.[134] El estómago del adulto en condiciones de ayuno presenta un pH de alrededor de 2,[133] pero este valor puede aumentar a más de 4 después de la ingesta de alimentos. Además, la acidez del estómago en niños es mucho menor, con un valor de pH de aproximadamente 4, lo que contribuiría a una menor y más lenta degradación de los alérgenos.[135] A la vista de nuestros resultados, es de esperar que ambos alérgenos, OVA y LYS, sobrevivan en cierta medida a la pepsinolisis *in vivo*, dependiendo este grado de resistencia del pH gástrico existente en cada circunstancia. Además de influir sobre la estructura de los alérgenos y su susceptibilidad enzimática, el

valor de pH tiene un efecto moderado sobre la actividad de la pepsina, que es óptima entorno a pH 2 y disminuye progresivamente a pHs superiores.[136]

La fosfatidilcolina (PC) es un fosfolípido secretado por la mucosa gástrica y presente también en la bilis y en la yema de huevo. Aparte de su función protectora de la mucosa, tiene propiedades tensioactivas, al tratarse una sustancia anfifílica, y es el principal componente de las bicapas lipídicas que forman las membranas celulares. En el presente trabajo se observó un ligero efecto protector de este fosfolípido en la degradación de la LYS por pepsina, similar al observado para la α-lactalbúmina (α-La) por otros autores y también reproducido en nuestros experimentos. Se ha descrito que la α-La adopta una conformación parcialmente desdoblada de "molten globule" a pH 2 que le permitiría penetrar en las vesículas de PC, hecho que retarda su proteolisis.[67] En el caso de la LYS, hemos observado que la adición de PC a pH ácido provoca un cambio de fluorescencia, indicativo de la existencia de un entorno proteico más hidrofóbico, que sugiere una interacción con las vesículas de PC. De hecho, se ha sugerido que la LYS podría ser atrapada por liposomas de PC.[137] LYS y α-La son dos proteínas homólogas que presentan muy diferente susceptibilidad a la acción de la pepsina. La α-La adquiere a pH 2 una conformación muy flexible que la hace muy susceptible al ataque enzimático. Por el contrario, y como ya se ha mencionado, la estructura terciaria de la LYS, según los espectros de dicroísmo, no se ve alterada a este pH, lo que explicaría su mayor resistencia a la pepsinolisis.

La presencia de PC no modificó la susceptibilidad de la OVA a la hidrólisis por pepsina. Como ya se ha indicado, el dicroísmo circular de la OVA a pH 2 corrobora que esta proteína también adopta una estructura de "molten globule", aunque probablemente bastante rígida debido al puente disulfuro intracatenario.[138] Una mayor exposición de los residuos hidrofóbicos favorecería su interacción con las vesículas de PC, tal y como sugiere la disminución de fluorescencia observada con la adición del fosfolípido. No obstante, la OVA probablemente no adquiere la flexibilidad suficiente para penetrar en las vesículas de PC, viéndose inalterada su susceptibilidad a la proteolisis por pepsina. Análogamente, se ha descrito que la digestión por pepsina de otras proteínas como la albúmina 2S de la nuez de

Brasil[139] o la β-Lg,[140] tampoco se ve afectada por la presencia de PC en fluido gástrico simulado de pH 2 ó 2.5.

Las sales biliares son moléculas anfifílicas cuya principal función es solubilizar los lípidos que resultan de la hidrólisis de los triglicéridos. En el medio duodenal, las sales biliares y la PC se asocian en solución formando micelas mixtas en las que las colas hidrofóbicas de las moléculas de PC se disponen radialmente hacia el interior, mientras que las moléculas de sales biliares se insertan longitudinalmente con la cara hidrofílica orientada hacia el disolvente.[141] La presencia de ambos surfactantes, en concentraciones típicas del estado pospandrial, favorece la hidrólisis enzimática de la OVA. A pH neutro, los ensayos de ELISA evidenciaron un menor reconocimiento de la OVA por los anticuerpos IgG, que podría indicar un posible cambio estructural inducido por las sales biliares. Por otro lado, a un pH superior al punto isoeléctrico de la proteína, la carga negativa propiciaría una interacción de tipo electrostático con la PC. De hecho, se ha propuesto que, en el duodeno, las proteínas podrían asociarse con las micelas de sales biliares y PC.[108] Estas interacciones podrían alterar la estructura de la OVA, de modo que expusiera nuevas regiones al ataque por las enzimas duodenales. Un reciente estudio sobre el efecto de las sales biliares en la digestión de algunas proteínas de la dieta, también reveló que la hidrólisis de proteínas como la β-Lg, la albúmina sérica bovina o la mioglobina se veía favorecida en presencia de estos compuestos.[142] Por tanto, resulta importante destacar el papel adicional que juegan las sales biliares durante la digestión gastrointestinal fomentando la proteolisis, además de la lipólisis.

Por su parte, la LYS experimentó un descenso brusco de solubilidad en el medio duodenal con adición de sales biliares, si bien la presencia de PC atenuó ligeramente esta pérdida de solubilidad. Sarkar y col.[107] observaron un comportamiento marcadamente distinto de las proteínas de la leche βLg (p.I.~5) y lactoferrina (p.I.~9) en emulsiones aceite:agua en presencia de sales biliares. Mientras que la primera era desplazada de la interfase al pH duodenal, la segunda se mantenía recubriendo las gotas de aceite y se unía mediante interacciones electrostáticas a las moléculas aniónicas de sales biliares. La LYS comparte con la lactoferrina el carácter altamente catiónico (p.I.~11), por lo que es muy probable que interaccione de forma similar con las sales biliares, lo que conduciría a una

reducción de su carga superficial. Si a ello sumamos que el pH duodenal está más próximo al punto isoeléctrico de la LYS, se puede argumentar que, en dichas condiciones, la ausencia de repulsión electrostática y las interacciones hidrofóbicas determinarían la agregación y precipitación proteica. Además, observamos que la precipitación dependía de la concentración de sales biliares en el medio. A baja concentración, característica del estado de ayuno,[105] detectamos aún proteína soluble, mientras que, en presencia de mayores concentraciones, típicas de condiciones intermedias o posprandiales ésta fue prácticamente nula. Solo la presencia de PC atenuó el efecto de las sales biliares, lo que podría deberse a una menor interacción de la LYS con las micelas mixtas.

En cualquier caso, la precipitación parcial del alérgeno a su llegada al intestino tendría varias implicaciones fisiológicas. Por un lado, la insolubilización podría dificultar el acceso de las proteasas a los lugares de corte específicos, quedando mayor proporción de proteína intacta y alterando la proporción de agentes antimicrobianos que alcancen la región inferior del intestino. Por otro lado, se conoce que los antígenos particulados son transportados preferentemente a través de los folículos linfoides, ruta que podrían seguir los agregados de LYS insolubles. Se ha descrito, por ejemplo, que la agregación de proteínas lácteas inducida por el calentamiento dificulta su transporte a través de los enterocitos para ser absorbidas vía placas de Peyer.[81] Esta ruta, además, se relacionó con un mayor riesgo de sensibilización al antígeno específico. Asimismo, una alta permeabilidad intestinal, como la que presentan niños de corta edad, debe considerarse un factor de riesgo de especial relevancia en el caso de la LYS, ya que podrían atravesar el epitelio elevadas cantidades de proteína intacta.

Mientras que el análisis de OVA y LYS por separado nos ha aportado información sobre la influencia concreta de factores tales como la relación ezima:sustrato, el pH o la concentración de surfactantes en el proceso digestivo, la extensión del estudio al huevo completo (*resultados 2.3*) permitió evaluar el efecto de los componentes de la matriz del alimento en la susceptibilidad de los alérgenos a la digestión y su posterior capacidad de unión a IgE. De hecho, diversos estudios han señalado la relevancia de la matriz del alimento en la digestibilidad de las proteínas. Por ejemplo, los polisacáridos en frutas o la presencia de proteínas con actividad inhibidora de tripsina en el cacahuete dificultan la

digestión de los alérgenos en sus respectivos alimentos.[143, 144] De modo similar, muchas proteínas alimentarias interaccionan con lípidos para formar emulsiones y otras estructuras, lo que modifica su susceptibilidad a la digestión.[81]

El estudio de la susceptibilidad de los principales alérgenos cuando se empleó clara de huevo como sustrato de las enzimas gástricas y duodenales reveló que, mientras que la ovotransferrina (OVT) era hidrolizada rápidamente, se detectaban OVA y LYS intactas en los digeridos gástricos y duodenales. Esto está en consonancia con los resultados de digestibilidad obtenidos para las proteínas individuales (*resultados 2.1 y 2.2*), si bien los resultados sugirieron una mayor resistencia de la OVA a las enzimas pancreáticas en la clara de huevo, que podría atribuirse a la presencia de ovomucoide (OM). El OM posee actividad inhibidora de tripsina y esta actividad la mantienen, aunque en menor medida, los productos de degradación que se forman debido su hidrólisis por la pepsina en el medio estomacal. [89, 145]

La presencia de la yema favorece ligeramente la hidrólisis de las proteínas de la clara, a juzgar por la mayor abundancia de productos de degradación observada. La débil influencia de los lípidos de la yema podría achacarse a que éstos se encuentran formando parte de lipoproteínas de baja densidad y no originan interfases donde podrían adsorberse los alérgenos y sufrir cambios en su susceptibilidad enzimática, tal y como les ocurre, por ejemplo, a la β-Lg y a la β-caseína de la leche en emulsiones aceite:agua.[66] No obstante, respecto a la LYS encontramos una diferencia importante: el digerido gastroduodenal de la mezcla de clara y yema contenía una cantidad muy elevada de alérgeno remanente, lo que sugiere que los componentes de la yema interfieren en su precipitación por las sales biliares. Este fenómeno podría deberse a la unión de las sales biliares a las lipoproteínas de baja densidad (LDL) de la yema, lo que reduciría el número de moléculas de surfactante disponibles para interaccionar con la LYS y evitaría su precipitación en el medio duodenal. Adicionalmente, y puesto que se había comprobado que la presencia de PC disminuía la precipitación inducida por las sales biliares (*resultados 2.2*), es de suponer que este fosfolípido, abundante en la yema, también contribuirá a que una mayor proporción de alérgeno permanezca soluble y sobreviva a la digestión gastrointestinal cuando se ingiere el huevo completo. La presencia de LYS intacta en los digeridos de clara y yema indica,

además, que esta proteína resiste la acción de las enzimas duodenales en el huevo completo.

Tras la digestión gastrointestinal *in vitro*, tanto OVA como LYS retienen reactividad frente a IgE de pacientes con alergia al huevo, puesto que su degradación no llega a ser completa. De hecho, observamos que los digeridos gástricos de LYS son tanto o más inmunorreactivos que la proteína sin digerir (*resultados 2.2*) que, por otra parte, presenta una elevadísima afinidad por IgE. Respecto a la OVA, los digeridos duodenales fueron menos inmunorreactivos (*resultados 2.1,*) aunque pudo comprobarse que un número importante de fragmentos derivados de esta proteína conservan sus epítopos íntegros (*resultados 2.3*). Además, detectamos unión a IgE en un péptido derivado de LYS presente en los digeridos gastroduodenales de clara de huevo. Asimismo, varios fragmentos fueron reconocidos por el anticuerpo policlonal frente a OM, confirmando su contribución a la inmunorreactividad del digerido gastroduodenal de clara. De hecho, la reactividad frente a IgE de los fragmentos de OM producidos en la digestión, tanto por pepsina como por tripsina y quimotripsina, ha sido demostrada por numerosos autores.[73, 89, 145] Las variaciones en la digestibilidad de los alérgenos OVA y LYS encontradas debido a las distintas condiciones ensayadas (presencia de PC, sales biliares o yema de huevo) no se tradujeron en variaciones significativas en la capacidad de unión a IgE de los hidrolizados obtenidos.

Estos resultados ponen de manifiesto que, no sólo la OVA y LYS intactas, sino también los péptidos producidos por su digestión, son inmunorreactivos frente a IgE. Poulsen y Hau.[146] estudiaron la inmunogenicidad de hidrolizados de proteínas de trigo, llegando a la conclusión de que tanto la proteína intacta como sus fragmentos mayores de 3.5 kDa eran capaces de unirse a la IgE de mastocitos y producir su desgranulación. Numerosos estudios posteriores han evidenciado la capacidad de fragmentos proteolíticos de reaccionar con la IgE del suero de individuos alérgicos. Por ejemplo, un péptido de 10 kDa producido durante la digestión del alérgeno del salmón, beta'-c,[147] o fragmentos de los alérgenos de la avellana (Cor a 1) o la manzana (Mal d 1), retienen su capacidad de unión a IgE.[148] No obstante, se ha de subrayar que la simple unión de IgE no implicaría la desgranulación de mastocitos y basófilos, ya que un alérgeno debe reaccionar con al menos

dos moléculas de IgE ancladas en la superficie de dichas células efectoras para producirse su activación.[47]

En la búsqueda de alérgenos potenciales minoritarios en la clara de huevo, se han identificado de forma tentativa dos proteínas, ovoinhibidor y clusterina, que reaccionaron con la IgE de individuos alérgicos al huevo (*resultados 2.3*) . Tras la hidrólisis enzimática, no se observó la persistencia de péptidos inmunorreactivos menores de 30 kDa procedentes de dichas proteínas, lo que sugiere que éstas pierden su inmunorreactividad tras la digestión gastrointestinal.

La proteína transportadora de riboflavina (RfBP) es una proteína minoritaria necesaria para transportar la riboflavina (vitamina B2) al oocito que juega un papel determinante en el desarrollo embrionario.[149] Se ha estudiado poco desde el punto de vista alergénico, aunque su elevada estabilidad (presenta 9 puentes disulfuro) podría conferirle resistencia a las enzimas digestivas a su paso por el tracto gastrointestinal, una característica que comparten muchos alérgenos alimentarios. De hecho, nuestro estudio pone de manifiesto, por primera vez, que la RfBP es reactiva frente a IgE de pacientes alérgicos al huevo y que los puentes disulfuro le proporcionan un cierto grado de integridad estructural tras la acción de la pepsina (*resultados 2.4*). En la etapa duodenal, la proteína se digiere completamente, originando péptidos con capacidad de unir IgE de pacientes alérgicos al huevo, al menos uno de los cuales se encuentra en el fragmento 41-84. El epítopo aquí contenido no comprende ninguna de las dos posiciones de glicosilación de la RfBP, Asn 36 y 147,[150] lo que sugiere que la unión a IgE se efectúa a través de la fracción proteica.

La RBP es una proteína minoritaria en la clara de huevo (0.8 %),[151] por lo que su contribución a la alergenicidad de los alimentos con huevo puede ser limitada. No obstante, estudios recientes han propuesto el uso de RBP como aditivo para inhibir el amargor de sustancias como la naringina o la cafeína,[152] por lo que habría que proceder con precaución antes de incorporarlo en grandes cantidades a determinados alimentos. Aunque la capacidad de unión a IgE no implica necesariamente alergenicidad, estos resultados deben catalogar a esta proteína como un alérgeno minoritario potencial, que podría tener la

capacidad de sensibilizar o desencadenar una respuesta alérgica en individuos susceptibles, incluso encontrándose en bajas concentraciones.

3.2 Interpretación bioquímica de la reducida alergenicidad de los alérgenos del huevo calentados

La mayor tolerancia hacia el huevo tratado térmicamente en relación al no tratado[78] ofrece una inmejorable oportunidad para investigar los factores de los que depende la alergenicidad de las proteínas. Es frecuente afirmar que el tratamiento térmico a que se someten los alérgenos durante el procesado conlleva la destrucción de los epítopos conformacionales.[153] Por tanto, los alérgenos que contienen preferentemente epítopos lineales, así como aquellos cuya estructura es termorresistente, retienen su inmunorreactividad. Aunque este argumento explicaría la reducción de alergenicidad en muchos casos, no es el único mecanismo posible. En el presente trabajo hemos aportado nuevos datos para comprender el mecanismo de interacción con el sistema inmunitario de los principales alérgenos del huevo en su forma calentada (*resultados 2.5*).

Cuando sometemos a la OVA a un calentamiento de 95 ^{o}C durante 30 min, sufre un proceso de desnaturalización y agregación que la hace muy susceptible a la hidrólisis enzimática. El tratamiento térmico de la OVA induce el desplegamiento parcial de la proteína, propiciando la interacción entre los residuos hidrofóbicos de varias moléculas que se dispondrían formando agregados con una estructura de lámina beta intermolecular.[154] Por tanto, dicha estructura dejaría accesibles múltiples lugares de corte específicos para la pepsina, que hidrolizaría más fácilmente las macromoléculas de proteína en comparación con la elevada resistencia que presenta la proteína nativa, ya mencionada. La acentuada hidrólisis enzimática de la OVA calentada también ha sido descrita por varios autores, estudiándola tanto individualmente como en la clara de huevo. Joo y Kato[155] observaron que la OVA tratada térmicamente era digerida completamente, siendo indetectable en el intestino o en la sangre de ratones que la habían ingerido, en contraposición a la OVA no tratada. En este trabajo se ha confirmado la pérdida de alergenicidad en ratones y, además, se ha demostrado, por primera vez en un ensayo funcional *in vitro* con basófilos humanos,

la reducida capacidad de la OVA, calentada y digerida simulando las condiciones gastrointestinales, de inducir desgranulación, lo que en una situación *in vivo* se traduciría en una menor capacidad de desencadenar los síntomas de una reacción anafiláctica.

Asimismo, la OVA calentada, a diferencia de la nativa, no es capaz de atravesar las células *Caco-2* y activar a los basófilos humanos, lo que sugiere que la porción de OVA calentada que pudiera llegar intacta al intestino no es absorbible, posiblemente a causa de la formación de los agregados intermoleculares. Además, las células T específicas de los ratones que ingirieron la proteína calentada no sufrieron activación, por lo que cabe suponer que los péptidos procedentes de la extensa hidrólisis de la OVA calentada tampoco son reconocidos por las células T. Estas hipótesis corroborarían la observación de que la OVA suministrada por vía parenteral sí produce síntomas en los ratones sensibilizados, ya que se elude el transporte intestinal.

El caso del OM es diferente, ya que se trata de una proteína termorresistente cuya, ya de por sí elevada, digestibilidad no se altera por el calentamiento. Diversos autores han señalado que el OM retiene su capacidad de unión a IgE tras la digestión[89, 145] y el tratamiento térmico.[73] Así, cabría esperar que ambas formas, tratada y no tratada, provocaran síntomas alérgicos y conservaran su capacidad de activación de basófilos. Sin embargo, nuestros resultados muestran que el calentamiento también repercute en la absorción intestinal del OM, ya que solo produce síntomas en los ratones que lo reciben vía parenteral. Hirose y col.[156] encontraron evidencias de que el calentamiento produce un cambio estructural irreversible del OM, apareciendo nuevos epítopos en su superficie. Puesto que el 90% de las proteínas absorbidas son degradadas en los compartimentos lisosómicos durante la transcitosis,[157] dicho cambio estructural podría determinar una mayor hidrólisis o un perfil peptídico distinto, carente de inmunorreactividad. Alternativamente, la forma calentada podría ser incapaz de atravesar los enterocitos. Ambas hipótesis serían factibles a la vista del experimento de estimulación de basófilos con la fracción basolateral recogida después de la incubación de las células Caco-2 con el OM calentado, ya que dicha fracción no produjo la activación de basófilos, un ensayo que presenta una buena correlación con la reactividad clínica.[158]

En este trabajo se pone de manifiesto que las modificaciones estructurales inducidas por procesos tecnológicos tienen un impacto a todos los niveles relacionados con el fenómeno alérgico. Es decir, influyen en la digestibilidad gastrointestinal de los alérgenos, la absorción intestinal y la presentación al sistema inmunitario como antígenos inmunológicamente activos o inactivos.

A pesar de que nuestros experimentos *in vivo* e *in vitro* descartan la alergenicidad de OVA y OM calentados, en humanos existe un porcentaje de individuos alérgicos (25% aproximadamente[78]) que reaccionan frente al huevo calentado. Urisu y col.[159] encontraron que prácticamente la totalidad de los pacientes que no toleraban la clara de huevo tratada térmicamente, sí lo hacían al eliminar el OM del alimento. Por tanto, es probable que en los individuos sensibles al huevo calentado, los fragmentos de OM absorbidos intestinalmente mantengan su alergenicidad.

Por último, merece la pena discutir la aparición en ciertas ocasiones de reacciones sistémicas pocos minutos después de la ingestión de un alimento alergénico.[160] Una posible explicación a este rápido brote de los síntomas es la existencia de absorción en la cavidad bucal. Dirks y col.[161] demostraron, mediante un ensayo de liberación de histamina por basófilos, la presencia de cacahuete en sangre 10 minutos después de introducirlo en la boca, masticarlo brevemente y escupirlo. Por tanto, los alérgenos pueden ser en algunos casos absorbidos pre-intestinalmente a través de la mucosa bucal en una forma inmunológicamente activa. Martínez y col.[162] encontraron que un 84% de las manifestaciones clínicas ocurrían en los primeros 30 minutos tras la ingestión de huevo. Es probable, pues, que los alérgenos del huevo también puedan ser parcialmente absorbidos por la mucosa bucal. Sería interesante llevar a cabo estudios adicionales en esta dirección para comprender más profundamente los fenómenos implicados en la respuesta alérgica o en la sensibilización en las etapas previas a la digestión gastrointestinal.

3.3 Desensibilización mediante inmunoterapia oral

Numerosas investigaciones han empleado protocolos de inmunoterapia oral que han logrado diferentes grados de éxito.[31, 32, 83] No obstante, los resultados de desensibilización suelen ser temporales y requieren una ingesta continuada del alimento para evitar la reaparición de la hipersensibilidad. Además, las reacciones adversas a menudo llevan a muchos pacientes a abandonar la inmunoterapia. Un conocimiento más profundo de los mecanismos implicados en el desarrollo de tolerancia posibilitaría implementar terapias más seguras y efectivas.

En este trabajo hemos observado que el protocolo de inmunoterapia ensayado en el modelo murino C3H/HeJ conduce a una protección clínica temporal (desensibilización) pero no al desarrollo de tolerancia (*resultados 2.6*). La desensibilización se produce a pesar de la reactividad de basófilos y mastocitos periféricos y la presencia de IgE en suero, y es independiente de la supresión generalizada de citoquinas observada en respuesta a la inmunoterapia. Estos datos están en contraposición con la teoría que sugiere que la inmunoterapia desgranula progresivamente basófilos y mastocitos hasta volverlos insensibles al alérgeno.[163] Skripak y col.[164] tampoco observaron inactivación de mastocitos tisulares durante la inmunoterapia oral en pacientes alérgicos a la leche, al no encontrar diferencias en las pruebas cutáneas entre el grupo tratado y el placebo. Asimismo, demostraron que la inmunoterapia oral no producía cambios en los niveles de IgE pese a conseguir una desensibilización efectiva. Análogamente, encontramos que los ratones que recibieron la inmunoterapia presentaban niveles elevados de IgE y, sin embargo, no reaccionaban al alérgeno.

Ciertos cambios en el perfil de citoquinas, como por ejemplo la producción de IL-10 por células Treg y la inversión del desequilibrio Th1/Th2, se han observado en distintas etapas de los protocolos de inmunoterapia.[165] Sin embargo, nuestros datos muestran una supresión de todas las citoquinas estudiadas como consecuencia de la inmunoterapia, sin que ello esté relacionado con la protección clínica.

En el presente estudio planteamos un mecanismo de protección localizado en la mucosa gastrointestinal y no sistémico, ya que los ratones tratados aún reaccionaban al alérgeno suministrado vía parenteral. La desensibilización podría implicar a las células epiteliales de la mucosa gastrointestinal, que mediarían un bloqueo de la detección o absorción del alérgeno. Así lo indicaría la reducción de la expresión de varios genes que se manifiestan principalmente en las células epiteliales y que están relacionados con enzimas digestivas y péptidos antimicrobianos. En cualquier caso, la asociación de estos genes con la protección clínica debe estudiarse más profundamente, así como la verosimilitud en humanos.

Recientemente, Kulis y col.[166] desensibilizaron ratones alérgicos a la nuez y al anacardo mediante inmunoterapia oral con uno solo de los frutos secos. Este resultado sugiere que la desensibilización obedece a un mecanismo inespecífico de antígeno, es decir, cualquier antígeno circunstante relacionado podría inducir la protección clínica. Nuestros resultados concuerdan con esta hipótesis, ya que la inmunoterapia con OM también produjo desensibilización frente a OVA en los ratones sensibilizados a ambos antígenos.

Por último, un resultado importante ha sido demostrar que el alérgeno OM calentado, incapaz de inducir una respuesta anafiláctica en ratones, mantiene su capacidad de desensibilización al administrarlo oralmente siguiendo una inmunoterapia. A pesar de que, como vimos anteriormente (*resultados 2.5*), el calentamiento produce cambios importantes en OVA y OM favoreciendo su hidrólisis enzimática y dificultando su absorción intestinal, la capacidad de actuar sobre las células epiteliales parece no verse comprometida, salvaguardándose el mecanismo de desensibilización. Si a ello unimos la baja inmunorreactividad de estos alérgenos calentados, se nos revela una aproximación mucho más segura para la inmunoterapia oral en individuos alérgicos al huevo, con menor incidencia de reacciones adversas.

4. CONCLUSIONES

CONCLUSIONES

1. El pH determina la susceptibilidad de los alérgenos OVA y LYS a la digestión por pepsina cuando ésta se emplea en concentraciones fisiológicas (172 U/mg de proteína). A pH 2 o superior, característico del estado posprandial y predominante en niños y personas con la función gástrica alterada, ambos alérgenos sobreviven en gran medida a la pepsinolisis.

2. La OVA adopta a pH 2 una estructura parcialmente desnaturalizada de "molten globule" que facilita su interacción con fosfatidilcolina, aunque no se ve alterada su susceptibilidad enzimática. A este pH, la interacción de la LYS con este fosfolípido dificulta su degradación por pepsina.

3. Al pH neutro duodenal, las sales biliares, la fosfatidilcolina y la combinación de ambos surfactantes, favorecen la digestión de la OVA por las enzimas duodenales. La LYS precipita en un medio duodenal simulado con presencia de concentraciones posprandiales de sales biliares, aunque la presencia de fosfatidilcolina reduce parcialmente este fenómeno.

4. OVA y LYS retienen su capacidad de unión a IgE tras la digestión gastrointestinal, tanto al evaluarlas como proteínas aisladas, como cuando están formando parte de la clara de huevo completa.

5. La reactividad frente a IgE de los digeridos gastroduodenales de clara de huevo reside mayoritariamente en péptidos procedentes de la OVA, además de en la presencia de OVA y LYS no degradadas, aunque no puede descartarse la contribución de productos de degradación del OM.

6. La yema no influye significativamente en la digestibilidad e inmunorreactividad de los alérgenos del huevo. No obstante, favorece que una mayor proporción de LYS

soluble permanezca en el medio duodenal al reducir la precipitación que provocan las sales biliares.

7. Dos proteínas minoritarias de la clara de huevo, identificadas como ovoinhibidor y clusterina, poseen la capacidad de reaccionar con la IgE de individuos alérgicos al huevo. La proteína transportadora de riboflavina, también minoritaria en la clara de huevo, posee la capacidad de unir IgE tanto en su forma intacta, como tras su digestión gastrointestinal *in vitro*. Dos péptidos resistentes a las enzimas digestivas contienen epítopos inmunorreactivos que pertenecen a la región Leu41 - Trp84 de la secuencia de la proteína.

8. La OVA tratada térmicamente pierde su alergenicidad en un modelo murino de alergia a alimentos como consecuencia de su mayor susceptibilidad a la digestión gastrointestinal y un transporte intestinal dificultado.

9. El tratamiento térmico del OM elimina su alergenicidad en el mismo modelo. Aunque su digestión gastrointestinal no se modifica, el alérgeno calentado pierde la capacidad de activar a las células efectoras tras el transporte intestinal, apuntando a una hidrólisis acentuada durante la transcitosis en los enterocitos como causa de su inocuidad.

10. La inmunoterapia usando OM calentado produce una desensibilización igualmente eficaz que empleando el alérgeno nativo, perfilándose el uso de alérgenos calentados como una aproximación terapéutica más segura para los individuos alérgicos al huevo.

11. La inmunoterapia oral con clara de huevo u OM durante 14 días produce desensibilización temporal pero no tolerancia en ratones sensibilizados a OVA u OM. La desensibilización ocurre a pesar de la existencia de niveles elevados de IgE en suero y origina una supresión generalizada de citoquinas en los esplenocitos

estimulados con el alérgeno que, sin embargo, no guarda relación con la protección clínica.

12. Los ratones desensibilizados mediante inmunoterapia oral reaccionan frente al alérgeno suministrado por vía parenteral, por lo que la desensibilización no estaría mediada por cambios en las células efectoras periféricas sino por un mecanismo localizado en la mucosa gastrointestinal y asociado a una reducción en la expresión de genes epiteliales.

5. BIBLIOGRAFÍA

BIBLIOGRAFÍA

En este apartado se incluyen las referencias citadas en la Introducción y en la Discusión General.

1. Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion in vitro. Nat Biotechnol 1996; 14:1269-73.
2. Besler M, Steinhut H, Paschke A. Stability of food allergens and allergenicity of processed foods. J Chromatogr B 2001; 756:207-28.
3. Kenna JG, Evans RM. Digestibility of proteins in simulated gastric fluid. Toxicologists 2000; 54:141.
4. Amo A, Rodriguez-Perez R, Blanco J, Villota J, Juste S, Moneo I, et al. Gal d 6 Is the Second Allergen Characterized from Egg Yolk. J Agric Food Chem 2010; 58:7453-7.
5. Branum AM, Lukacs SL. Food allergy among children in the United States. Pediatrics 2009; 124:1549-55.
6. Gupta R, Sheikh A, Strachan DP, Anderson HR. Time trends in allergic disorders in the UK. Thorax 2007; 62:91-6.
7. Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, et al. The prevalence of food allergy: a meta-analysis. J Allergy Clin Immunol 2007; 120:638-46.
8. Sicherer SH, Leung DY. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects in 2010. J Allergy Clin Immunol 2011; 127:326-35.
9. Sicherer SH, Sampson HA. Food allergy. J Allergy Clin Immunol 2006; 117:S470-5.
10. Brandtzaeg P. Food allergy: separating the science from the mythology. Nat Rev Gastro Hepat 2010; 7:380-400.
11. Lack G. Epidemiologic risks for food allergy. J Allergy Clin Immunol 2008; 121:1331-6.
12. Bjorksten B. The Hygiene Hypothesis: Do We Still Believe in It? Microbial-Host Interaction: Tolerance Versus Allergy. Nestle Nutr Workshop Ser Pediatr Program 2009; 64:11-22.
13. Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukocyte Biol 2011; 89:685-95.
14. Nowak-Wegrzyn A, Sampson HA. Adverse reactions to foods. Med Clin North Am 2006; 90:97-127.
15. Bischoff SC. Physiological and pathophysiological functions of intestinal mast cells. Semin Immunopathol 2009; 31:185-205.
16. Lack G, Fox D, Northstone K, Golding J. Factors associated with the development of peanut allergy in childhood. N Engl J Med 2003; 348:977-85.
17. Fernandez-Rivas M, Bolhaar S, Gonzalez-Mancebo E, Asero R, van Leeuwen A, Bohle B, et al. Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods. J Allergy Clin Immunol 2006; 118:481-8.

18. Untersmayr E, Bakos N, Scholl I, Szalai K, Scheiner O, Stahl-Skov P, et al. Gastric acid suppression supports sensitization to dietary proteins and lowers threshold levels. J Invest Dermatol 2005; 125:A50-A50.
19. Yang PC, Jury J, Soderholm JD, Sherman PM, McKay DM, Perdue MH. Chronic psychological stress in rats induces intestinal sensitization to luminal antigens. Am J Pathol 2006; 168:104-14.
20. Watson CJ, Rowland M, Warhurst G. Functional modeling of tight junctions in intestinal cell monolayers using polyethylene glycol oligomers. Am J Physiol Cell Physiol 2001; 281:C388-97.
21. Rescigno M, Rotta G, Valzasina B, Ricciardi-Castagnoli P. Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology 2001; 204:572-81.
22. Heyman M, Ducroc R, Desjeux JF, Morgat JL. Horseradish-Peroxidase Transport across Adult-Rabbit Jejunum Invitro. Am J Physiol 1982; 242:G558-64.
23. Mallegol J, Van Niel G, Lebreton C, Lepelletier Y, Candalh C, Dugave C, et al. T84-intestinal epithelial exosomes bear MHC class II/peptide complexes potentiating antigen presentation by dendritic cells. Gastroenterology 2007; 132:1866-76.
24. Bevilacqua C, Montagnac G, Benmerah A, Candalh C, Brousse N, Cerf-Bensussan N, et al. Food allergens are protected from degradation during CD23-mediated transepithelial transport. Int Arch Allergy Immunol 2004; 135:108-16.
25. Berin MC, Shreffler WG. T(H)2 adjuvants: Implications for food allergy. J Allergy Clin Immunol 2008; 121:1311-20.
26. Segal DM, Taurog JD, Metzger H. Dimeric Immunoglobulin-E Serves as a Unit Signal for Mast-Cell Degranulation. Proc Nat Acad Sci USA 1977; 74:2993-97.
27. Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol 2005; 115:3-12.
28. Bamboat ZM, Stableford JA, Plitas G, Burt BM, Nguyen HM, Welles AP, et al. Human Liver Dendritic Cells Promote T Cell Hyporesponsiveness. J Immunol 2009; 182:1901-11.
29. Berin MC, Mayer L. Immunophysiology of experimental food allergy. Mucosal Immunol 2009; 2:24-32.
30. Nowak-Wegrzyn A, Sampson HA. Future therapies for food allergies. J Allergy Clin Immunol; 127:558-73.
31. Zapatero L, Alonso E, Fuentes V, Martinez MI. Oral Desensitization in Children With Cow's Milk Allergy. J Investig Allergol Clin Immunol 2008; 18:389-96.
32. Patriarca G, Nucera E, Roncallo C, Pollastrini E, Bartolozzi F, De Pasquale T, et al. Oral desensitizing treatment in food allergy: clinical and immunological results. Aliment Pharmacol Ther 2003; 17:459-65.
33. Enrique E, Malek T, Pineda F, Palacios R, Bartra J, Tella R, et al. Sublingual immunotherapy for hazelnut food allergy: a follow-up study. Ann Allergy Asthma Immunol 2008; 100:283-4.
34. Dupont C, Kalach N, Soulaines P, Legoue-Morillon S, Piloquet H, Benhamou PH. Cow's milk epicutaneous immunotherapy in children: a pilot trial of safety, acceptability, and impact on allergic reactivity. J Allergy Clin Immunol; 125:1165-7.
35. Rupa P, Mine Y. Ablation of ovomucoid-induced allergic response by desensitization with recombinant ovomucoid third domain in a murine model. Clin Exp Immunol 2006; 145:493-501.

36. Li XM, Srivastava K, Grishin A, Huang CK, Schofield B, Burks W, et al. Persistent protective effect of heat-killed Escherichia coli producing "engineered," recombinant peanut proteins in a murine model of peanut allergy. J Allergy Clin Immunol 2003; 112:159-67.
37. Kim JS, Nowak-Wegrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA. Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol; 128:125-31 e2.
38. Konstantinou GN, Giavi S, Kalobatsou A, Vassilopoulou E, Douladiris N, Saxoni-Papageorgiou P, et al. Consumption of heat-treated egg by children allergic or sensitized to egg can affect the natural course of egg allergy: hypothesis-generating observations. J Allergy Clin Immunol 2008; 122:414-5.
39. Leung DY, Sampson HA, Yunginger JW, Burks AW, Jr., Schneider LC, Wortel CH, et al. Effect of anti-IgE therapy in patients with peanut allergy. N Engl J Med 2003; 348:986-93.
40. Srivastava KD, Qu C, Zhang T, Goldfarb J, Sampson HA, Li XM. Food Allergy Herbal Formula-2 silences peanut-induced anaphylaxis for a prolonged posttreatment period via IFN-gamma-producing CD8+ T cells. J Allergy Clin Immunol 2009; 123:443-51.
41. Lee SY, Huang CK, Zhang TF, Schofield BH, Burks AW, Bannon GA, et al. Oral administration of IL-12 suppresses anaphylactic reactions in a murine model of peanut hypersensitivity. Clin Immunol 2001; 101:220-8.
42. Creticos PS, Chen YH, Schroeder JT. New approaches in immunotherapy: allergen vaccination with immunostimulatory DNA. Immunol Allergy Clin North Am 2004; 24:569-81.
43. Kalliomaki M, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet 2003; 361:1869-71.
44. Arslanoglu S, Moro GE, Schmitt J, Tandoi L, Rizzardi S, Boehm G. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. J Nutr 2008; 138:1091-5.
45. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol 2008; 121:847-52.
46. Hoffmann-Sommergruber K, Mills ENC. Food allergen protein families and their structural characteristics and application in component-resolved diagnosis: new data from the EuroPrevall project. Anal Bioanal Chem 2009; 395:25-35.
47. Huby RDJ, Dearman RJ, Kimber I. Why are some proteins allergens? Toxicol Sci 2000; 55:235-46.
48. Untersmayr E, Bakos N, Scholl I, Kundi M, Roth-Walter F, Szalai K, et al. Anti-ulcer drugs promote IgE formation toward dietary antigens in adult patients. FASEB J 2005; 19:656-8.
49. Hogan SP, Mishra A, Brandt EB, Royalty MP, Pope SM, Zimmermann N, et al. A pathological function for eotaxin and eosinophils in eosinophilic gastrointestinal inflammation. Nat Immunol 2001; 2:353-60.
50. Barone KS, Reilly MR, Flanagan MP, Michael JG. Abrogation of oral tolerance by feeding encapsulated antigen. Cell Immunol 2000; 199:65-72.

51. Buchanan BB, Adamidi C, Lozano RM, Yee BC, Momma M, Kobrehel K, et al. Thioredoxin-linked mitigation of allergic responses to wheat. Proc Natl Acad Sci U S A 1997; 94:5372-7.
52. del Val G, Yee BC, Lozano RM, Buchanan BB, Ermel RW, Lee YM, et al. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. J Allergy Clin Immunol 1999; 103:690-7.
53. Breiteneder H, Mills C. Nonspecific lipid-transfer proteins in plant foods and pollens: an important allergen class. Curr Opin Allergy Clin Immunol 2005; 5:275-79.
54. Chitpinityol S, Crabbe MJC. Chymosin and aspartic proteinases. Food Chem 1998; 61:395-418.
55. Dominguez J, Cuevas M, Urena V, Munoz T, Moneo I. Purification and characterization of an allergen of mustard seed. Ann Allergy 1990; 64:352-7.
56. Murtagh GJ, Archer DB, Dumoulin M, Ridout S, Matthews S, Arshad SH, et al. In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. Clin Exp Allergy 2003; 33:1147-52.
57. Scheurer S, Lauer I, Foetisch K, San Miguel Moncin M, Retzek M, Hartz C, et al. Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. J Allergy Clin Immunol 2004; 114:900-7.
58. Maleki SJ, Kopper RA, Shin DS, Park CW, Compadre CM, Sampson H, et al. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. J Immunol 2000; 164:5844-9.
59. Foetisch K, Westphal S, Lauer I, Retzek M, Altmann F, Kolarich D, et al. Biological activity of IgE specific for cross-reactive carbohydrate determinants. J Allergy Clin Immunol 2003; 111:889-96.
60. Gu JX, Matsuda T, Nakamura R, Ishiguro H, Ohkubo I, Sasaki M, et al. Chemical Deglycosylation of Hen Ovomucoid - Protective Effect of Carbohydrate Moiety on Tryptic Hydrolysis and Heat Denaturation. J Biochem 1989; 106:66-70.
61. Creamer LK. Effect of Sodium Dodecyl-Sulfate and Palmitic Acid on the Equilibrium Unfolding of Bovine Beta-Lactoglobulin. Biochemistry 1995; 34:7170-76.
62. Douliez JP, Jegou S, Pato C, Molle D, Tran V, Marion D. Binding of two mono-acylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modelling. Eur J Biochem 2001; 268:384-8.
63. Valenta R, Bugajska-Schretter A, Grote M, Vangelista L, Valent P, Sperr WR, et al. Purification, biochemical, and immunological characterisation of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin. Gut 2000; 46:661-69.
64. Mills ENC, Sancho AI, Rigby NM, Jenkins JA, Mackie AR. Impact of food processing on the structural and allergenic properties of food allergens. Mol Nutr Food Res 2009; 53:963-9.
65. Aguilera JM, Parada J. Food microstructure affects the bioavailability of several nutrients. J Food Sci 2007; 72:R21-32.
66. Macierzanka A, Sancho AI, Mills ENC, Rigby NM, Mackie AR. Emulsification alters simulated gastrointestinal proteolysis of beta-casein and beta-lactoglobulin. Soft Matter 2009; 5:538-50.

67. Moreno FJ, Mackie AR, Mills ENC. Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. J Agric Food Chem 2005; 53:9810-16.
68. Rosch P, Neudecker P, Schweimer K, Nerkamp J, Scheurer S, Vieths S, et al. Allergic cross-reactivity made visible - Solution structure of the major cherry allergen Pru av 1. J Biol Chem 2001; 276:22756-63.
69. Kohno Y, Honma K, Saito K, Shimojo N, Tsunoo H, Kaminogawa S, et al. Preferential Recognition of Primary-Protein Structures of Alpha-Casein by IgG and IgE Antibodies of Patients with Milk Allergy. Ann Allergy 1994; 73:419-22.
70. Curioni A, Simonato B, Pasini G, Giannattasio M, Peruffo ADB, De Lazzari F. Food allergy to wheat products: The effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. J Agric Food Chem 2001; 49:5668-73.
71. Maleki SJ, Chung SY, Champagne ET, Raufman JP. The effects of roasting on the allergenic properties of peanut proteins. J Allergy Clin Immunol 2000; 106:763-68.
72. Corzo-Martinez M, Soria AC, Belloque J, Villamiel M, Moreno FJ. Effect of glycation on the gastrointestinal digestibility and immunoreactivity of bovine beta-lactoglobulin. Int Dairy J 2010; 20:742-52.
73. Jimenez-Saiz R, Belloque J, Molina E, Lopez-Fandino R. Human Immunoglobulin E (IgE) Binding to Heated and Glycated Ovalbumin and Ovomucoid before and after in Vitro Digestion. J Agric Food Chem 2011; 59:10044-51.
74. Sampson HA. Food sensitivity and the pathogenesis of atopic dermatitis. J R Soc Med 1997; 90:2-8.
75. Uguz A, Lack G, Pumphrey R, Ewan P, Warner J, Dick J, et al. Allergic reactions in the community: a questionnaire survey of members of the anaphylaxis campaign. Clin Exp Allergy 2005; 35:746-50.
76. Savage JH, Matsui EC, Skripak JM, Wood RA. The natural history of egg allergy. J Allergy Clin Immunol 2007; 120:1413-17.
77. Urisu A, Ando H, Morita Y, Wada E, Yasaki T, Yamada K, et al. Allergenic activity of heated and ovomucoid-depleted egg white. J Allergy Clin Immunol 1997; 100:171-6.
78. Lemon-Mule H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, Nowak-Wegrzyn A. Immunologic changes in children with egg allergy ingesting extensively heated egg. J Allergy Clin Immunol 2008; 122:977-83.
79. Jarvinen KM, Beyer K, Vila L, Bardina L, Mishoe M, Sampson HA. Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. Allergy 2007; 62:758-65.
80. Konstantinou GN, Giavi S, Kalohatsou A, Hassilopoulou E, Douladiris N, Saxoni-Papageorgiou P, et al. Consumption of heat-treated egg by children allergic or sensitized to egg can affect the natural course of egg allergy: Hypothesis generating observations. J Allergy Clin Immunol 2008; 122:414-15.
81. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy 2008; 63:882-90.
82. Beyer K, Wahn U. Oral immunotherapy for food allergy in children. Curr Opin Allergy Clin Immunol 2008; 8:553-6.

83. Buchanan AD, Green TD, Jones SM, Scurlock AM, Christie L, Althage KA, et al. Egg oral immunotherapy in nonanaphylactic children with egg allergy. J Allergy Clin Immunol 2007; 119:199-205.
84. Mine Y, Rupa P. Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin. Prot Eng 2003; 16:747-52.
85. Zhang JW, Mine Y. Characterization of IgE and IgG epitopes on ovomucoid using egg-white-allergic patients' sera. Biochem Biophys Res Commun 1998; 253:124-27.
86. Zhang JW, Mine Y. Characterization of residues in human IgE and IgG binding site by chemical modification of ovomucoid third domain. Biochem Biophys Res Commun 1999; 261:610-3.
87. Holen E, Bolann B, Elsayed S. Novel B and T cell epitopes of chicken ovomucoid (Gal d 1) induce T cell secretion of IL-6, IL-13, and IFN-gamma. Clin Exp Allergy 2001; 31:952-64.
88. Mine Y, Yang M. Recent advances in the understanding of egg allergens: basic, industrial, and clinical perspectives. J Agric Food Chem 2008; 56:4874-900.
89. Kovacs-Nolan J, Zhang JW, Hayakawa S, Mine Y. Immunochemical and structural analysis of pepsin-digested egg white ovomucoid. J Agric Food Chem 2000; 48:6261-6.
90. Takagi K, Teshima R, Okunuki H, Sawada J. Comparative study of in vitro digestibility of food proteins and effect of preheating on the digestion. Biol Pharm Bull 2003; 26:969-73.
91. Mine Y, Zhang JW. Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. J Agric Food Chem 2002; 50:2679-83.
92. Langeland T. A Clinical and Immunological Study of Allergy to Hens Egg-White. Allergy 1983; 38:399-412.
93. Holen E, Elsayed S. Specific T cell lines for ovalbumin, ovomucoid, lysozyme and two OA synthetic epitopes, generated from egg allergic patients' PBMC. Clin Exp Allergy 1996; 26:1080-8.
94. Polverino de Laureto P, Frare E, Gottardo R, van Dael H, Fontana A. Partly folded states of members of the lysozyme/lactalbumin superfamily: A comparative study by circular dichroism spectroscopy and limited proteolysis. Prot Sci 2002; 11:2932-46.
95. Quirce S, Maranon F, Umpierrez A, de las Heras M, Fernandez-Caldas E, Sastre J. Chicken serum albumin (Gal d 5*) is a partially heat-labile inhalant and food allergen implicated in the bird-egg syndrome. Allergy 2001; 56:754-62.
96. Chambers SJ, Wickham MSJ, Regoli M, Bertelli E, Gunning PA, Nicoletti C. Rapid in vivo transport of proteins from digested allergen across pre-sensitized gut. Biochem Biophys Res Commun 2004; 325:1258-63.
97. Paganelli R, Levinsky RJ. Solid-Phase Radioimmunoassay for Detection of Circulating Food Protein Antigens in Human-Serum. J Immunol Methods 1980; 37:333-41.
98. Husby S, Jensenius JC, Svehag SE. Passage of Undegraded Dietary Antigen into the Blood of Healthy-Adults - Further Characterization of the Kinetics of Uptake and the Size Distribution of the Antigen. Scan J Immunol 1986; 24:447-55.
99. Untersmayr E, Poulsen LK, Platzer MH, Pedersen MH, Boltz-Nitulescu G, Skov PS, et al. The effects of gastric digestion on codfish allergenicity. J Allergy Clin Immunol 2005; 115:377-82.

100. Fu TT, Abbott UR, Hatzos C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - A comparative study. J Agric Food Chem 2002; 50:7154-60.
101. Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, et al. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regul Toxicol Pharmacol 2004; 39:87-98.
102. Gomes RAD, Batista RP, de Almeida AC, da Fonseca DN, Juliano L, Hial V. A fluorimetric method for the determination of pepsin activity. Anal Biochem 2003; 316:11-4.
103. Moreno FJ. Gastrointestinal digestion of food allergens: Effect on their allergenicity. Biomed Pharmacother 2007; 61:50-60.
104. Tropea JE, Nashed NT, Louis JM, Sayer JM, Jerina DM. Effect of Salt on the Kinetic-Parameters of Retroviral and Mammalian Aspartic-Acid Proteases. Bioorg Chem 1992; 20:67-76.
105. Dressman JB, Amidon GL, Reppas C, Shah VP. Dissolution testing as a prognostic tool for oral drug absorption: Immediate release dosage forms. Pharm Res 1998; 15:11-22.
106. Schmidt DG, Meijer RJGM, Slangen CJ, Vanberesteijn ECH. Raising the Ph of the Pepsin-Catalyzed Hydrolysis of Bovine Whey Proteins Increases the Antigenicity of the Hydrolysates. Clin Exp Allergy 1995; 25:1007-17.107.
107. Sarkar A, Horne DS, Singh H. Interactions of milk protein-stabilized oil-in-water emulsions with bile salts in a simulated upper intestinal model. Food Hydrocol 2010; 24:142-51.
108. Burnett GR, Wickham M, Fillery-Travis A, Robertson JA, Belton PS, Gilbert SM, et al. Interaction between protein allergens and model gastric emulsions. Biochem Soc Trans 2002; 30:916-8.
109. Dufour E, Dalgalarrondo M, Adam L. Conformation of beta-lactoglobulin at an oil/water interface as determined from proteolysis and spectroscopic methods. J Colloid Interface Sci 1998; 207:264-72.
110. Wickham M, Faulks R, Mills C. In vitro digestion methods for assessing the effect of food structure on allergen breakdown. Mol Nutr Food Res 2009; 53:952-58.
111. Caillard I, Tome D. Transport of Beta-Lactoglobulin and Alpha-Lactalbumin in Enterocyte-Like Caco-2 Cells. Reprod Nutr Dev 1995; 35:179-88.
112. Curtis GH, Gall DG. Macromolecular Transport by Rat Gastric-Mucosa. Am J Physiol 1992; 262:G1033-40.
113. Tsume Y, Taki Y, Sakane T, Nadai T, Sezaki H, Watabe K, et al. Quantitative evaluation of the gastrointestinal absorption of protein into the blood and lymph circulation. Biol Pharmaceut Bull 1996; 19:1332-7.
114. Weangsripanaval T, Moriyama T, Kageura T, Ogawa T, Kawada T. Dietary fat and an exogenous emulsifier increase the gastrointestinal absorption of a major soybean allergen, Gly m Bd 30K, in mice. J Nutr 2005; 135:1738-44.
115. Pinto M, Robineleon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, et al. Enterocyte-Like Differentiation and Polarization of the Human-Colon Carcinoma Cell-Line Caco-2 in Culture. Biol Cell 1983; 47:323-30.
116. Zweibaum A, Hauri HP, Sterchi E, Chantret I, Haffen K, Bamat J, et al. Immunohistological Evidence, Obtained with Monoclonal-Antibodies, of Small

Intestinal Brush-Border Hydrolases in Human-Colon Cancers and Fetal Colons. Int J Cancer 1984; 34:591-8.

117. Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev 2001; 46:27-43.
118. Hilgendorf C, Spahn-Langguth H, Regardh CG, Lipka E, Amidon GL, Langguth P. Caco-2 versus Caco-2/HT29-MTX co-cultured cell lines: Permeabilities via diffusion, inside- and outside-directed carrier-mediated transport. J Pharmaceut Sci 2000; 89:63-75.
119. Mahler GJ, Shuler ML, Glahn RP. Characterization of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability. J Nutr Biochem 2009; 20:494-502.
120. Choi SH, Nishikawa M, Sakoda A, Sakai Y. Feasibility of a simple double-layered coculture system incorporating metabolic processes of the intestine and liver tissue: application to the analysis of benzo[a]pyrene toxicity. Toxicol In Vitro 2004; 18:393-402.
121. Siebers A, Finlay BB. M cells and the pathogenesis of mucosal and systemic infections. Trends Microbiol 1996; 4:22-9.
122. Liang E, Kabcenell AK, Coleman JR, Robson J, Ruffles R, Yazdanian M. Permeability measurement of macromolecules and assessment of mucosal antigen sampling using in vitro converted M cells. J Pharmacol Toxicol Methods 2001; 46:93-101.
123. Rytkonen J, Valkonen KH, Virtanen V, Foxwell RA, Kyd JM, Cripps AW, et al. Enterocyte and M-cell transport of native and heat-denatured bovine beta-lactoglobulin: Significance of heat denaturation. J Agric Food Chem 2006; 54:1500-7.
124. Grundy J, Matthews S, Bateman B, Dean T, Arshad SH. Rising prevalence of allergy to peanut in children: Data from 2 sequential cohorts. J Allergy Clin Immunol 2002; 110:784-9.
125. Hu Y, Chen J, Li H. Comparison of food allergy prevalence among Chinese infants in Chongqing, 2009 versus 1999. Pediatr Int; 52:820-4.
126. Rudders SA, Banerji A, Vassallo MF, Clark S, Camargo CA, Jr. Trends in pediatric emergency department visits for food-induced anaphylaxis. J Allergy Clin Immunol; 126:385-8.
127. Amigo-Benavent M, Clemente A, Ferranti P, Caira S, del Castillo MD. Digestibility and immunoreactivity of soybean beta-conglycinin and its deglycosylated form. Food Chem 2011; 129:1598-605.
128. Untersmayr E, Vestergaard H, Malling HJ, Jensen LB, Platzer MH, Boltz-Nitulescu G, et al. Incomplete digestion of codfish represents a risk factor for anaphylaxis in patients with allergy. J Allergy Clin Immunol 2007; 119:711-7.
129. Taylor SL. Comment on digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - A comparative study. J Agric Food Chem 2003; 51:5183-4.
130. Dearman RJ, Caddick H, Stone S, Kenna JG, Basketter DA, Kimber I. Immunogenic properties of rapidly digested food proteins following gavage exposure of mice: a comparison of ovalbumin with a potato acid phosphatase preparation. Food Chem Toxicol 2002; 40:625-33.
131. Pellegrini A. Antimicrobial peptides from food proteins. Curr Pharm Des 2003; 9:1225-38.

132. Koldovski O. Developing of human gastrointestinal functions: interaction of changes in diet composition, hormonal maturation and fetal genetic programming. J Am Coll Nutr 1984; 3:131-8.
133. Tyssandier V, Reboul E, Dumas JF, Bougteloup-Demange C, Armand M, Marcand J, et al. Processing of vegetable-borne carotenoids in the human stomach and duodenum. Am J Physiol Gastrointest Liver Physiol 2003; 284:G913-23.
134. Untersmayr E, Schoell I, Foerster-Waldl E, Beil JW, Riemer A, Boltz-Nitulescu G, et al. Antacid medication preserves proteins from gastric digestion and may lead to food allergy. Pediatr Res 2003; 53:13a-13a.
135. Schmidt E, Edenkohler J, Tonkaboni F, Tolle J. Alimentary allergy prevention in infants with familial increased allergic risk: The effect of different feeding regimens in the first 6 months on atopic manifestations during the first year of life - A large-scale feeding trial. Nestlé Nutrition Workshop *Intestinal Immunology and Food Allergy*. Raven Press. 1995; 34:231-48.
136. Schlamowitz M, Peterson LU. Studies on the Optimum Ph for the Action of Pepsin on Native and Denatured Bovine Serum Albumin and Bovine Hemoglobin. J Biol Chem 1959; 234:3137-45.
137. Witoonsaridsilp W, Panyarachun B, Sarisuta N, Muller-Goymann CC. Influence of microenvironment and liposomal formulation on secondary structure and bilayer interaction of lysozyme. Colloid Surf B Biointerfaces 2010; 75:501-9.
138. Tatsumi E, Yoshimatsu D, Hirose M. Conformational state of disulfide-reduced ovalbumin at acid pH. Biosci Biotechnol Biochem 1999; 63:1285-90.
139. Moreno FJ, Mellon FA, Wickham MSJ, Bottrill AR, Mills ENC. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. FEBS J 2005; 272:341-52.
140. Mandalari G, Mackie AM, Rigby NM, Wickham MS, Mills EN. Physiological phosphatidylcholine protects bovine beta-lactoglobulin from simulated gastrointestinal proteolysis. Mol Nutr Food Res 2009; 53 Suppl 1:S131-9.
141. Hjelm RP, Thiyagarajan P, Alkanonyuksel H. Organization of Phosphatidylcholine and Bile-Salt in Rodlike Mixed Micelles. J Phys Chem 1992; 96:8653-61.
142. Gass J, Vora H, Hofmann AF, Gray GM, Khosla C. Enhancement of dietary protein digestion by conjugated bile acids. Gastroenterology 2007; 133:16-23.
143. Polovic N, Blanusa M, Gavrovic-Jankulovic M, Atanaskovic-Markovic M, Burazer L, Jankov R, et al. A matrix effect in pectin-rich fruits hampers digestion of allergen by pepsin in vivo and in vitro. Clin Exp Allergy 2007; 37:764-71.
144. Maleki SJ, Viquez O, Jacks T, Dodo H, Champagne ET, Chung SY, et al. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. J Allergy Clin Immunol 2003; 112:190-5.
145. Takagi K, Teshima R, Okunuki H, Itoh S, Kawasaki N, Kawanishi T, et al. Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. Int Arch Allergy Immunol 2005; 136:23-32.
146. Poulsen OM, Hau J. Murine passive cutaneous anaphylaxis test (PCA) for the 'all or none' determination of allergenicity of bovine whey proteins and peptides. Clin Allergy 1987; 17:75-83.
147. Fujita S, Shimizu Y, Kishimura H, Watanabe K, Hara A, Saeki H. In vitro digestion of major allergen in salmon roe and its peptide portion with proteolytic resistance. Food Chem 2012; 130:644-50.

148. Sancho AI, Wangorsch A, Jensen BM, Watson A, Alexeev Y, Johnson PE, et al. Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: Impact on structure and allergenic activity. Mol Nutr Food Res; 55:1690-9.
149. Adiga PR. Riboflavin carrier protein in reproduction. *In* Vitamin Receptors: Vitamins as Ligands in Cell Communication (Dakshinamurti, K., Ed.) 1994:137-176. Cambridge University Press, UK.
150. Amoresano A, Brancaccio A, Andolfo A, Perduca M, Monaco HL, Marino G. The carbohydrates of the isoforms of three avian riboflavin-binding proteins. Eur J Biochem 1999; 263:849-58.
151. White HB, Armstrong J, Whitehead CC. Riboflavin-Binding Protein - Concentration and Fractional Saturation in Chicken Eggs as a Function of Dietary Riboflavin. Biochem J 1986; 238:671-5.
152. Maehashi K, Matano M, Nonaka M, Udaka S, Yamamoto Y. Riboflavin-binding protein is a novel bitter inhibitor. Chem Senses 2008; 33:57-63.
153. Nowak-Wegrzyn A, Fiocchi A. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. Curr Opin Allergy Clin Immunol 2009; 9:234-7.
154. Kato A, Takagi T. Formation of Intermolecular Beta-Sheet Structure during Heat Denaturation of Ovalbumin. J Agric Food Chem 1988; 36:1156-9.
155. Joo K, Kato Y. Assessment of allergenic activity of a heat-coagulated ovalbumin after in vivo digestion. Biosci Biotechnol Biochem 2006; 70:591-7.
156. Hirose J, Kitabatake N, Kimura A, Narita H. Recognition of native and/or thermally induced denatured forms of the major food allergen, ovomucoid, by human IgE and mouse monoclonal IgG antibodies. Biosci Biotechnol Biochem 2004; 68:2490-7.
157. Heyman M, Ducroc R, Desjeux JF, Morgat JL. Horseradish peroxidase transport across adult rabbit jejunum in vitro. Am J Physiol 1982; 242:G558-64.
158. Moneret-Vautrin DA, Sainte-Laudy J, Kanny G, Fremont S. Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy. Ann Allergy Asthma Immunol 1999; 82:33-40.
159. Urisu A, Ando H, Morita Y, Wada E, Yasaki T, Yamada K, et al. Allergenic activity of heated and ovomucoid-depleted egg white. J Allergy Clin Immunol 1997; 100:171-6.
160. Wensing M, Penninks AH, Hefle SL, Koppelman SJ, Bruijnzeel-Koomen CAFM, Knulst AC. The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. J Allergy Clin Immunol 2002; 110:915-20.
161. Dirks CG, Pedersen MH, Platzer MH, Bindsley-Jensen C, Skov PS, Poulsen LK. Does absorption across the buccal mucosa explain early onset of food-induced allergic systemic reactions? J Allergy Clin Immunol 2005; 115:1321-3.
162. Martinez TB, Garcia-Ara C, Diaz-Pena JM, Munoz FM, Sanchez GG, Esteban MM. Validity of specific IgE antibodies in children with egg allergy. Clin Exp Allergy 2001; 31:1464-9.
163. Castells M. Rapid Desensitization for Hypersensitivity Reactions to Medications. Immunol Allergy Clin North Am 2009; 29:585-606.
164. Skripak JM, Nash SD, Rowley H, Brereton NH, Oh S, Hamilton RG, et al. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. J Allergy Clin Immunol 2008; 122:1154-60.

165. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 2006; 117:433-42.
166. Kulis M, Li YF, Lane H, Pons L, Burks W. Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts. J Allergy Clin Immunol 2011; 127:81-8.